THE EFFECT OF FUNCTIONAL ELECTRICAL STIMULATION ON OLIGODENDROCYTE BIOLOGY AFTER SPINAL CORD INJURY

by

Amber Tahnia Ballard

A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, MD
March, 2014

Abstract

In the developing and adult central nervous system (CNS), oligodendrocyte (OL) biology is controlled intrinsically by transcription and epigenetic factors, and extrinsically by neural activity and signaling molecules. Neural activity regulates OL development through the release of neurotransmitters and soluble molecules such as glutamate, ATP, and LIF. The epigenome controls gene expression through DNA methylation and post-transcriptional repression by microRNAs (miRs). These signaling pathways are dysregulated following CNS injury or disease, making them potential targets for therapeutic intervention. However, the effect of patterned neural activity on OL development and epigenetic mechanisms after spinal cord injury (SCI) is not fully understood. We found that the application of functional electrical stimulation (FES) after a complete mid-thoracic SCI in adult rats enhances proliferation of oligodendrocyte progenitor cells (OPCs) in the lumbar spinal cord in a frequency-dependent manner. Our data show that SCI alters miR expression caudal to the lesion level, and that FES treatment delays the injury-induced repression of OL-specific miR-338 and miR-23a. Furthermore, FES promotes the upregulation of miR-19b and DNA methyltransferases 1 and 3b, which are associated with neurogenesis and oligodendrogenis in the CNS. Taken together, these results suggest that neural activity regulates OL development in the injured spinal cord by modulating epigenetic mechanisms.

Approved by:

Advisor: John McDonald, MD, PhD

Reader: Andres Hurtado, MD

Dedication

This dissertation and my work are dedicated to my family and friends who have supported me throughout this journey. A special thanks to my parents for their endless love, support, and encouragement, for which I am eternally grateful.

Acknowledgements

The guidance and support of numerous individuals aided me in the completion of this work. Thank you all.

Andres Hurtado

Anna Miglioretti

John McDonald

Andrea Benedict

Visar Belegu

Deborah Castillo

Devin Gary

Tessa Ropp

Dane Wendell

Thierry Houdayer

Sahar Rahiem

Mollie Lange

Craig Remenapp

James Pendleton

Edward Hammond

Victoria Ohanna

Table of Contents

List of Figures

Chapter 1: Introduction

1.1 Spinal Cord Injury Pathology

Traumatic spinal cord injury (SCI) occurs to approximately 12,000 people in the United States every year, and the number of people with SCI that are currently alive is projected to increase to over 276,000 by 2014 (Devivo, 2012). The average age at the time of injury is 37.1 years, and men are injured 3-4 times more often than women. Overall, the leading cause of traumatic SCI is still motor vehicle crashes (Devivo, 2012), followed by accidents at work and during sports or recreation.

In traumatic SCI, the major injury types include impact with and without compression, laceration, transection, or distraction. The most common injury mechanism in humans is impact plus persistent compression (Dumont et al., 2001). Tissue movement as a result of impact occurs predominantly at the center of the spinal cord, shearing cell membranes and severely injuring axons near the gray matter (GM). The surface of the spinal cord experiences the least movement, allowing axons near the pia to survive and form a subpial axonal rim (Profyris et al., 2004). Myelinated axons are also extremely vulnerable to stretching from tissue movement because the longitudinal forces are concentrated at nodes of Ranvier (Profyris et al., 2004).

The initial mechanical disruption of the spinal cord is followed by a progressive secondary injury cascade that destroys local and distant nervous tissue (Dumont et al., 2001; Norenberg, Smith, & Marcillo, 2004; Profyris et al., 2004). These degenerative mechanisms can prevent the transmission of neural activity across the lesion, resulting in permanent sensory, motor, and autonomic dysfunction. The secondary injury cascade consists of 3 overlapping phases: acute, sub-acute, and chronic.

1.1.1 Acute Phase

The acute phase occurs from seconds to the first few days after the injury. It is characterized by vascular damage, cellular membrane damage, inflammation and aberrant release of neurotransmitters in the extracellular space (Ahn, Lee, & Kang, 2006; Bramlett & Dietrich, 2007; Dumont et al., 2001). Vascular damage includes hemorrhage and edema, resulting in decreased blood flow and pressure-induced ischemia. Excessive glutamate release causes excitotoxicity by activating NMDA and AMPA/kainite signaling, leading to apoptosis of neurons and oligodendrocytes (OLs) and the initiation of Wallerian degeneration (WD) and demyelination (McDonald, Althomsons, Hyrc, Choi, & Goldberg, 1998; McDonald, Shapiro, Silverstein, & Johnston, 1998; Profyris et al., 2004). This first wave of apoptosis starts within a few hours post–injury (PI) and continues for several days in the lesion epicenter (Profyris et al., 2004). Oligodendrocyte progenitor cells (OPCs), which go on to differentiate into mature myelinating OLs, migrate to the injury site and proliferate extensively to replace the lost OLs,

reaching peak numbers within 2 days after injury (Barnabé-Heider et al., 2010; Rabchevsky, Sullivan, & Scheff, 2007). Microglia become activated, while leukocytes and macrophages invade and accumulate around the lesion epicenter (Bramlett & Dietrich, 2007; Norenberg et al., 2004).

1.1.2 Sub-acute Phase

The sub-acute phase, beginning a few days post-injury (DPI) and lasting for several weeks, includes revascularization, removal of necrotic debris, prominent glial responses, and a second wave of cell death. An increase in the number of blood vessel returns blood flow to the injured tissue. Activated microglia continue to secrete both protective (TNFα, IL-1β, IL-6) and harmful (glutamate, cytokines, nitric oxide, proteases, superoxide anions) molecules, while macrophages phagocytize debris from the first wave of necrosis and apoptosis (Norenberg et al., 2004). A glial scar starts to form around the lesion epicenter, initially consisting of myelin debris, blood born macrophages, and microglia. Astrocytes become activated at the edge of the lesion, sending out longer and thicker processes that will eventually become part of the astroglial scar (Fawcett & Asher, 1999; Profyris et al., 2004; Yuan & He, 2013). A second wave of apoptosis begins both rostral and caudal to the level of the lesion, occurring primarily in the WM, exacerbating axonal degeneration and demyelination (Bramlett & Dietrich, 2007; Profyris et al., 2004). In contrast, decreased apoptotic cell death in the injury epicenter, combined with repopulation of OLs in spared WM tracts, results in diminished levels of primary demyelination by 7 DPI

(Rabchevsky et al., 2007; Totoiu & Keirstead, 2005). The progressive increase in mature OLs during this time period is paralleled by a significant recovery of hind limb motor function in rat models of traumatic SCI, suggesting that remyelination of spared axons contributes to functional recovery. However, newly formed myelin remains morphologically distinct from normal myelin, resulting in axons with lower G-ratios (the ratio of the thickness of the myelin sheath to the diameter of the axon) and increased distance between internodes (Griffiths & McCulloch, 1983), possibly limiting complete functional recovery.

1.1.3 Chronic Phase

In the weeks to months and years after traumatic SCI, a cyst forms in the lesion epicenter and replaces the injured spinal cord tissue. Representing the final phase of the necrotic process, the fluid-filled cyst does not cause a problem clinically, but it is a poor substrate for axonal regeneration. Both an astroglial and a mesenchymal scar surround the cyst, isolating it from spared tissue. Processes from activated astrocytes become tightly interwoven to form the astroglial scar, resulting in an impenetrable physical barrier to axonal regeneration in animal models of SCI (Fawcett & Asher, 1999; Karimi-Abdolrezaee & Billakanti, 2012; Wanner et al., 2013; Yuan & He, 2013). In contrast, astroglial scar formation is much milder in humans and acts as a molecular barrier by secreting growth-inhibitory molecules such as myelin-associated glycoprotein (MAG), myelin oligodendroyte glycoprotein (MOG), chondroitin sulfate proteoglycan (CSPG), and Nogo (Fawcett & Asher, 1999;

Norenberg et al., 2004). It is the mesenchymal scar, composed of collagen and fibrous connective tissue,that provides an impenetrable physical barrier to regenerating axons in SCI patients.

Oligodendrocyte remyelination continues in the chronic phase, peaking at 10 weeks after injury in adult rats (Totoiu & Keirstead, 2005). Although newly formed myelin remains morphologically distinct from normal myelin, there is a significant positive correlation between remyelination and the recovery of function PI (Duncan, Brower, Kondo, Curlee, & Schultz, 2009). Therefore, therapeutic interventions that augment and stabilize regenerative mechanisms such as remyelination could result in substantially improved outcomes after SCI.

1.2 Current Strategies for Spinal Cord Repair

1.2.1 Overview

Numerous barriers must be overcome to restore function after CNS injury, including the replacement of lost tissue, scar removal, relief from inhibitory molecules, growth and guidance of regenerating axons, and remyelination of both spared and regenerated axons (Belegu, Oudega, Gary, & McDonald, 2007). Therapeutic interventions, such as cell transplantation (reviewed by J. Li and Lepski 2013), pharmacological intervention (reviewed by Wilson, Forgione, and Fehlings 2013; Bradbury and Carter 2011), biomaterials (reviewed by Krishna et al. 2013), epigenetic modulation (reviewed by Madathil et al. 2011; MacDonald

and Roskams 2009), activity-based therapies (Belegu et al., 2007), and functional electrical stimulation (Sadowsky et al., 2013) could attenuate secondary damage and augment regenerative mechanisms, resulting in substantially improved outcomes after SCI.

1.2.2 Functional Electrical Stimulation

Previous work has shown that electrical stimulation (ESTIM) of nerve tissue can improve activity-dependent processes. ESTIM enhances myelination by OLs and Schwann cells (SCs), the myelinating cells of the peripheral nervous system (PNS), when they are co-cultured with dorsal root ganglia (Ishibashi et al., 2006; Malone et al., 2013; B Stevens, Tanner, & Fields, 1998). OL survival is increased after stimulation of mixed cortical cultures (Gary et al. 2012). Unilateral stimulation of the medullary pyramid in adult rats promotes OPC proliferation and differentiation in the contralateral corticospinal tract (CST) (Q. Li, Brus-Ramer, Martin, & McDonald, 2010). Improved axonal regeneration and remyelination are observed in the crush-injured sciatic nerves of adult rats after just one hour of ES (Wan, Xia, & Ding, 2010). Additionally, short-term stimulation of the intact sciatic nerve in adult rats augments regeneration of its injured central projections into the lesion site after a chronic dorsal funiculotomy (Udina et al., 2008).

SCI results in decreased neural activity below the injury level, possibly limiting the spinal cord's endogenous repair potential (Dietz, 2010, 2012; Edgerton et al., 2001). Functional electrical stimulation (FES) returns patterned neural activity to

the injured spinal cord by activating peripheral nerves and generating functional movement of extremities that are paralyzed as a result of nervous system injury. When used as a rehabilitative therapy, FES can ameliorate muscle atrophy (Griffin et al., 2009; Sadowsky et al., 2013), increase muscle strength (Davis, Hamzaid, & Fornusek, 2008; Johnston, Modlesky, Betz, & Lauer, 2011) and endurance (Davis et al., 2008), enhance aerobic metabolism (Hamzaid, Pithon, Smith, & Davis, 2012), lessen spasticity (Sadowsky et al., 2013), promote intralimb coordination (Field-Fote & Tepavac, 2002), and improve both sensory and motor function in patients with CNS injury (Griffin et al., 2009; Sadowsky et al., 2013). In rodent models of SCI, FES treatment improves interlimb coordination (Jung, Belanger, Kanchiku, Fairchild, & Abbas, 2009), locomotion, and neurovascular response after an acute spinal cord contusion (Beaumont et al., 2013). It also promotes proliferation of neural progenitors cells after chronic spinal cord transection (Becker, Gary, Rosenzweig, Grill, & McDonald, 2010). However, the cellular and molecular mechanisms underlying the beneficial effects of FES therapy are not fully understood.

1.2.3 Epigenetic Modulation

Cellular development in the CNS is regulated intrinsically by communication between transcription factors and epigenetic factors (MacDonald & Roskams, 2009). The epigenome, which includes microRNAs (miRs), DNA methyltransferases (DNMTs), and histone deacetylases (HDACs), controls gene expression without affecting DNA sequences, possibly in response to external

stimuli (Morrison, Rodgers, Morgan, & Bale, 2013). Dsyregulation of the epigenome underlies aberrant CNS function in mental retardation, psychiatric disorders, and CNS injury (MacDonald & Roskams, 2009), making it a likely target for therapeutic intervention.

1.2.3.1 microRNAs

MicroRNAs (miRs) are small non-coding RNAs, approximately 23 nucleotides long, that base pair to the 3' untranslated region (3'UTR) of protein-coding gene mRNA. They control genes post-transcriptionally by inhibiting mRNA translation or targeting the mRNA for degradation (Dugas & Notterpek, 2011; He, Yu, Awatramani, & Lu, 2011). A miR only needs to be partially complementary to the target messenger RNA, allowing for the regulation of multiple target genes simultaneously. A specific network of miRs regulates gene expression in OL lineage cells during nervous system development (Barca-mayo & Lu, 2012). This network is dysregulated after CNS injury, possibly limiting endogenous regeneration and functional recovery (N.-K. Liu, Wang, Lu, & Xu, 2009; Yunta et al., 2012).

CNS injury results in dysregulation of the miR network, particularly the one that controls OL biology. In rodent models of acute SCI, these alterations are evident within and extending beyond the lesion epicenter (N.-K. Liu et al., 2009; Strickland et al., 2011; Yunta et al., 2012). The vast majority of miRs are downregulated at 1 DPI, and continue to decrease for up to a week after injury (N.-K. Liu et al., 2009; Strickland et al., 2011; Yunta et al., 2012). This trend is

also observed in OL-specific miRs, including miR-9, 138, 338*, 219-5p, and 219-2-3p (N.-K. Liu et al., 2009; Yunta et al., 2012), possibly limiting the extent of endogenous remyelination after injury. miR-17 and miR-20a of the 17-92 cluster are suppressed at the lesion site 3 days after a contusion injury, but return to control levels by 7 DPI (Yunta et al., 2012). In contrast, miRs-17 and 92a are elevated at both 1 and 7 DPI (N.-K. Liu et al., 2009).

microRNAs have also been implicated in the pathophysiology following traumatic brain injury (TBI), multiple sclerosis (MS), and ischemic stroke (Bhalala, Srikanth, & Kessler, 2013; J.-S. Li & Yao, 2012; N.-K. Liu & Xu, 2011; Madathil et al., 2011). TBI in adult rats results in the upregulation of miRs-199a, 17-5p, 20a, and 23a*, while suppressing miRs-9, 19b, 138, and 338 (Z. Hu et al., 2012; Lei, Li, Chen, Yang, & Zhang, 2009). Elevated levels of miR-92a were observed the in the plasma of mild and severe TBI patients, making it a potential biomarker for diagnosing TBI in humans (Redell, Moore, Ward, Hergenroeder, & Dash, 2010). Stroke elevates expression of the miR-17-92 cluster (Xian Shuang Liu et al., 2013), while repressing miR-124a (Xian Shuang Liu et al., 2011) in the subventricular zone (SVZ) of adult mice. It also downregulates miR-9 in ischemic white matter OPCs and OLs, leading to enhanced levels of serum response factor (SRF), a transcription factor that mediates OL differentiation (Buller et al., 2012). MS lesions, both active and inactive, have elevated levels of miR-214 and miR-23a, possibly reflecting endogenous attempts at remyelination (Junker et al., 2009). In contrast, miR-219 and miR-338 are heavily repressed in

chronically demyelinated lesions, which would limit the ability of OLs to differentiate and synthesize myelin (Junker et al., 2009).

Several studies have observed enhanced cellular regeneration and functional recovery after CNS injury by altering miR expression patterns, suggesting the potential use of miRs as therapeutic tools. The neuroprotective gene neurogenin 1 (Ngn1), which increases neurogenesis and motor neuron survival after traumatic SCI, can be upregulated by the inhibition of miR-20a of the miR-17-92 cluster (Jee, Jung, Im, Jung, & Kang, 2012). Knockdown of miR-486 promotes functional motor recovery by enhancing the expression of proneural factor NeuroD6 (Jee, Jung, Choi, et al., 2012). Systemic delivery of neural stem/progenitor cells (NSPCs) that overexpress miR-124 in contused rats enhances neuronal differentiation, at the expense of astroglial differentiation, decreases the lesion cavity volume, and stimulates functional recovery compared to control animals and those injected with normal NSPCs (Xu, Wang, Li, Qin, & Jiang, 2012). miR-124 can also be used to prevent persistent hyperalgesia and mechanical allodynia (Willemen et al., 2012). Infusion of miR-23b improves neuropathic pain in the injured spinal cord and restores GABA-mediated inhibitory mechanisms by preventing the death of GABAergic neurons (Im et al., 2012). Another potential therapeutic target is miR-21, which is upregulated over time following acute SCI (Bhalala et al., 2012; J.-Z. Hu et al., 2013). Its overexpression in astrocytes suppresses their typical hypertrophic response to SCI, resulting in a glial scar that's more permissive to axonal regeneration into the lesion site (Bhalala et al., 2012). Global inhibition of miR-21 after SCI is

detrimental to the recovery of hindlimb motor function, tissue sparing, and lesion size (J.-Z. Hu et al., 2013), illustrating the need to modulate miR expression in a cell-specific manner after CNS injury to maximize protective and regenerative mechanisms.

Exercise can also be used to modulate miR activity in the injured spinal cord. Following a complete spinal cord transection at vertebral level T10, five days of passive hind limb cycling per week increases miR-21 expression in the lumbar spinal cord, resulting in suppressed PTEN signaling (G. Liu, Detloff, Miller, Santi, & Houlé, 2012; G. Liu, Keeler, Zhukareva, & Houlé, 2010). In contrast, exercise blocks expression of miR-15b and miR-199a-3p, causing enhanced mTOR and Bcl-2 signaling, respectively. Cycling exercise also ameliorates the injury-induced increase in caspase-7 and -9 mRNA levels (G. Liu et al., 2010). Taken together, these finding suggest that activity-dependent changes in miR expression can promote axonal regeneration and neuronal plasticity, as well as hinder apoptotic cell death, in the injured spinal cord.

1.2.3.2 DNA Methylation

DNMTs catalyze the addition of methyl groups to cytosine residues in DNA, almost exclusively within cytosine-phosphate-guanine (CpG) rich domains (reviewed by MacDonald and Roskams 2009). Methyl-CpG-binding domain (MBD) proteins then bind to the methylated DNA, ultimately resulting in suppression of gene transcription. DNA methylation enzymes can be divided into 2 classes, maintenance (DNMT1) and *de novo* DNMTs (DNMT3a, DNMT3b).

Maintenance DNMTs preserve methylation patterns in daughter cells by adding methyl groups to partially-methylated CpGs during DNA replication, while *de novo* DNMTs create new methylation patterns by adding methyl groups to unmethylated CpGs. Expression of all 3 DNMTs can be found throughout the postnatal and adult CNS (Simmons, Stringfellow, Glover, Wagle, & Clinton, 2013), and they are required for normal CNS development (MacDonald & Roskams, 2009).

The effect of SCI on DNA methylation is not fully understood. However, the changes in DNMT activity observed after other types of CNS injury and disease suggests that DNMTs may be involved in the pathophysiology observed after traumatic SCI. Alterations in DNA methylation have been implicated in TBI, ischemic stroke, amyotrophic lateral sclerosis (ALS), and schizophrenia. Traumatic brain injury in adult rats decreases global DNA methylation both within and adjacent to the lesion site during the first 48 hours after injury, with microglia and macrophages being the major source of hypomethylated cells (Z.-Y. Zhang, Zhang, Fauser, & Schluesener, 2007). DNMT1, which is predominantly expressed in the nucleus of proliferating cells and the cytoplasm of mature neurons, is upregulated in the nucleus and cytoplasm of astrocytes after TBI, suggesting that DNA methylation plays a role in the astrocytic response to CNS injury (Lundberg et al., 2009). The maintenance DNMT is associated with the delayed death of hippocampal neurons after transient cerebral ischemia in gerbils (Lee et al., 2013), and the complete elimination or reduction of DNMT1 in post-mitotic neurons confers resistance to ischemia-induced TBI (MacDonald &

Roskams, 2009). The gene for the glial-specific potassium channel Kir4.1 is methylated by DNMT1, resulting in decreased Kir4.1 expression in epilepsy, ALS, ischemic brain injury, and SCI (Nwaobi, Lin, Peramsetty, & Olsen, 2014). Aberrant levels of DNMT3a also underlie the elevated motor neuron degeneration observed in human ALS patients (Martin & Wong, 2013). Increased methylation of the Sox10 gene, a mediator of terminal OL differentiation, represses OL gene expression in patients suffering from schizophrenia (Iwamoto et al., 2005).

To our knowledge, only one study has investigated to role of DNMTs in SCI repair (Iskandar et al., 2010). The authors used a spinal cord regeneration model (SCRM) in which adult mice received a combined bilateral dorsal column transection SCI and a unilateral sciatic nerve conditioning lesion. This injury model suppressed the expression of both *de novo* DNMTs and decreased overall spinal cord methylation. Folic acid treatment eliminated the injury-induced dysregulation of DNMT3a and DNMT3b, thereby restoring spinal cord methylation to pre-injury levels and promoting regeneration of sensory axons (Iskandar et al., 2010). Inhibiting DNMT3a activity protects motor neurons from apoptosis in vitro and in a mouse model of ALS (Martin & Wong, 2013).

1.2.3.3 Histone Modification

Acetylation of lysine residues in the N-terminal tails of histone proteins modulates the ability of histones to interact with DNA (reviewed by MacDonald and Roskams 2009). Gene expression is promoted by hyperacetylation, which is

catalyzed by histone acetylases (HATs), and repressed by hypoacetylation, catalyzed by HDACs. Proteins with HDAC activity can be broken up into several classes: class I (HDAC1, 2, 3, and 8), class II (HDAC4, 5, 6, 7, 9, 10), class III (SIRT2 and sirtuin), and class IV (HDAC11) (MacDonald & Roskams, 2009). Acetylation homeostasis, which is maintained by a balance of HAT and HDAC activity, is dysregulated in SCI, ischemia, TBI, and demyelinating diseases, resulting in the global suppression of histone acetylation and neurological deficits (Shein & Shohami, 2011). Administration of HDAC inhibitors after CNS injury can limit cell death and degeneration, as well as promote functional recovery, by restoring the HDAC-HAT balance, suggesting that HDACs are a potential therapeutic target for spinal cord repair.

Reduced histone acetylation is observed within 24 hours after traumatic SCI in adult rats, continuing for up to 2 weeks post-injury (Lv et al., 2011). Treatment with the HDAC inhibitor valproic acid (VPA) immediately or 8 hours after injury has a neuroprotective effect, decreasing lesion volume and preventing apoptotic cell death by upregulating the neuroprotective genes heat shock protein (Hsp70), B-cell lymphoma 2 (Bcl-2), brain-derived (BDNF) and glial-derived neurotrophic factor (GDNF), resulting in accelerated functional recovery (Lv et al., 2011; Lv, Han, Sun, Wang, & Dong, 2012). Intraspinal delivery of VPA also reduces the injury-induced inflammatory response by downregulating expression of the purinergic receptor P2X4 in activated microglia (Lu et al., 2013).

In rodent models of ischemic stroke, HDAC1/2 levels decline drastically in the lesion core, but are upregulated in astrocytes located in the adjacent subcortical

white matter (SCWM) (Baltan, Bachleda, Morrison, & Murphy, 2011). Increased HDAC1 expression is also found in adjacent striatal neurons, while HDAC2 is elevated in the cortex, SCWM, and subventricular zone. Treatment with VPA within 24 hours of a mild middle cerebral artery occlusion (MCAO) in rats promotes OL survival, oligodendrogenesis, and neurogenesis up to 1 month after injury (X S Liu et al., 2012). Repression of HDAC activity with suberoylanilide hydroxamic acid (SAHA) stabilizes ATP levels and reduces glutamate-mediated excitotoxicity when administered before or after oxygen and glucose deprivation (OGD) in optic nerves isolated from adult mice, preserving white matter function and axonal recovery (Baltan, Murphy, Danilov, Bachleda, & Morrison, 2011). Hypoacetylation after TBI is associated with the robust activation of inflammatory microglia in the injured hippocampus, which can attenuated by systemic delivery of the benzamide class of HDAC inhibitors (Shein & Shohami, 2011; B. Zhang et al., 2008). Treatment with VPA enhances motor function and spatial memory by reducing lesion volume, decreasing neuronal damage, and improving the integrity of the blood brain barrier (BBB) (Dash et al., 2010).

Acetylation homeostasis plays a critical role in CNS white matter injury and repair. Endoplasmic reticulum (ER) stress after contusive SCI causes the accumulation of the pro-apoptotic factor CHOP, resulting in axonal degeneration and demyelination (Penas et al., 2011). Application of VPA decreases CHOP expression, thereby increasing OL survival and promoting locomotor recovery. Treatment with VPA prior to or after the induction of experimental autoimmune encephalomyelin (EAE) in adult rats promotes remyelination by recruiting NSPCs

and OPCs to the lesion site (Pazhoohan et al., 2014). Inactivation of the Nbn gene, which underlies Nijmegen breakage syndrome, induces HDAC1/2 suppression in callosal OPCs, causing increased OPC apoptosis and a severe myelination deficiency (B. Liu, Chen, Wang, & Tong, 2014).

Following cuprizone-induced demyelination in the corpus callosum of 8 week old mice, HDAC1 is recruited to the promoter regions of the transcription factors Sox2 and Hes5 in OPCs (Shen, Sandoval, et al., 2008). The HDAC-mediated suppression of these OL differentiation inhibitors precedes the upregulation of Olig1, a transcription factor that is critical for remyelination, and the myelin proteins CNP and MAG, resulting in spontaneous remyelination. However, there is an age-dependent decline in HDAC recruitment after injury, which allows transcriptional inhibitors to accumulate in OL lineage cells and thereby limits the extent of spontaneous remyelination. This epigenetic memory loss can be recapitulated by the administration of the HDAC inhibitor valproic acid (VPA) during cuprizone treatment in young mice or in cultures of differentiating OPCs.

Chapter 2: Extrinsic and Intrinsic Control of Oligodendrocyte Biology

The oligodendrogenic developmental program is regulated by both extrinsic and intrinsic mechanisms. Transcription factors, chromatin remodeling, DNA methylation, and miRs control oligodendrocyte (OL) biology intrinsically (Zuchero & Barres, 2013). They play a role in every phase of OL biology, and often work together to regulate the various steps in OL development. Extrinsic activity-dependent communication between neurons and glia is also crucial in regulating oligodendrocyte progenitor cell (OPC) proliferation, OL differentiation, and myelin synthesis. Previous work has demonstrated that neuronal activity can act on oligodendroglial cells both directly, through OL-axon synapses, and indirectly *via* soluble molecules released from axons and neighboring astrocytes.

2.1 Origin and Lineage Specification

2.1.1 Extrinsic Control

During CNS development, neuroepithelial cells in the neural tube give rise to motor neurons, glia, and interneurons (Figure 2.1). Motor neurons (MNs) are generated in the ventral progenitor domain (pMN), and their production is complete by embryonic day 10.5 (E10.5) in mice (Fancy, Chan, Baranzini,

Franklin, & Rowitch, 2011). Beginning around E12.5, the floor and roof plates of the neural tube secrete the morphogens sonic hedgehog (SHH) and bone morphogenetic proteins (BMPs), respectively, triggering the switch from neuronal lineage specification to the generation of glial-restricted progenitors referred to as oligodendrocyte-type 2 astrocyte (O-2A) cells. SHH signaling upregulates the transcription factors (TFs) Olig1/2 and Nkx6, promoting the first wave of OPC specification in the pMN and V0-V3 domains of the ventral neural tube (Mitew et al., 2013). BMP signaling has the opposite effect, strongly inducing astroglial differentiation at the expense of oligdodendroglial fate by suppressing Olig1/2 via inhibitors of DNA binding (Id2/4) and the canonical Wnt/β-cantenin pathway (Rivera et al., 2010). A second wave of OPCs, independent of SHH signaling and Nkx6, arises from the dorsal neural tube starting at E15.5 (Cai et al., 2005; Fogarty, Richardson, & Kessaris, 2005). Dorsally-derived OPCs instead depend on fibroblast growth factor (FGF) to induce Olig-mediated lineage specification. OL lineage specification in the developing spinal cord also depends on the secretion of platelet-derived growth factor (PDGF), which promotes Olig2 expression by activating the PDGF receptor α (PDFGRα) (Rivera et al., 2010). Despite their embryonic origins, both groups of OPCs have the same electrical properties (Tripathi et al., 2011), and together these cells will mature to form the majority of OL lineage cells in the spinal cord (Fu et al., 2002).

2.1.2 Intrinsic Control

Intrinsic regulation of OL lineage specification occurs *via* epigenetic-mediated suppression of neural stem/progenitor cells (NSPCs) proliferation and alternative cell lineage progression (Figure 2.1). Neural progenitors are maintained in an undifferentiated state by BAF53a, a subunit of the Swi-Snf-like neural progenitor-specific BAF complex, and the transcription factor Sox2 (Shen & Casaccia-Bonnefil, 2008; Yoo, Staahl, Chen, & Crabtree, 2009). microRNAs-9/9* and -124 promote the transition of proliferating NSPCs into post-mitotic cells by repressing of BAF53a in an activity-dependent process (Yoo et al., 2009). Overexpression of these in cultures of mouse embryonic stem cells inhibits signal transducer and activator of transcription (STAT) 3 signaling, leading to reduced astrocyte lineage specification (Krichevsky, Sonntag, Isacson, & Kosik, 2006). miR-124 also relieves inhibition of neuronal lineage progression by suppressing Sox9 (Cheng, Pastrana, Tavazoie, & Doetsch, 2009; Farrell, Power, & McDermott, 2011), REST/Scp1 (F. Ji, Lv, & Jiao, 2013; Visvanathan, Lee, Lee, Lee, & Lee, 2007), and Ptbp1 (Makeyev, Zhang, Carrasco, & Maniatis, 2007). The proneural factors Isl1, NeuroD1, Otx2, FoxJ3 and ZFP238, which stimulate NSPC differentiation into neurons, are blocked by OL-specific miRs-219 and -338 (Dugas et al., 2010; Zhao et al., 2010).

Histone deacetylation plays a major role in OL lineage specification. Addition of SHH to primary cultures of P1 rat cortical OPCs promotes HDAC1/2 activity, favoring OL lineage progression at the expense of astrogenesis (M. Wu et al., 2012). Both histone deacetylases prevent the binding of β-cantenin to the Wnt

pathway mediator TCF7L2, thereby relieving inhibition of Olig1/2 expression (Ye et al., 2009). Blocking HDAC activity in cultured OPCs with trichostatin A (TSA), a class I HDAC inhibitor, promotes their conversion into NSPC-like cells that can give rise to all 3 CNS lineages (Lyssiotis et al., 2007). This lineage reversion requires acetylation of the promoter region of Sox2, a TF responsible for maintaining NSPCs in an undifferentiated state, in addition to signaling by FGF. In contrast, addition of TSA to embryonic NSPC cultures induces neuronal differentiation and suppresses astrocyte differentiation without affecting oligodendrogenesis (Balasubramaniyan et al., 2006; Huiling Liu et al., 2012). Oligodendrocyte lineage specification in the developing rodent forebrain is delayed by postnatal treatment with the HDAC inhibitor valproic acid (VPA) (Shen, Li, & Casaccia-Bonnefil, 2005). Valproic acid-mediated inhibition of HDAC activity in adult hippocampal NSPCs has a similar effect, inducing neurogenesis at the expense of oligodendrogenesis and astroglialgenesis by upregulating the expression of the transcription factor NeuroD (Hsieh, Nakashima, Kuwabara, Mejia, & Gage, 2004).

The function of DNA methylation in the differentiation of NSPCs into OL lineage cells has yet to be determined. However, DNMT activity is critical to the developmental switch from neurogenesis to astrogliogenesis (Juliandi, Abematsu, & Nakashima, 2010). The maintenance DNA methyltransferase, DNMT1, is upregulated in the nucleus of replicating cells in the developing CNS (MacDonald & Roskams, 2009). Expression of DNMT1 in NSPCs induces hypermethylation of the promoter for glial fibrillary acidic protein (GFAP), a gene

critical for astrocyte differentiation, preventing STAT3/SMAD binding and inhibiting the JAK/STAT signaling pathway (Fan et al., 2005). Conditional deletion of DNMT1 in NSPCs shifts neural development toward astrogliogenesis. The *de novo* DNA methyltransferases, DNMT3a and 3b, are also required for neurogenesis. Upregulation of DNMT3a/3b levels with folic acid induces neuronal lineage specification in neurospheres derived from neonatal rat NSPCs (Luo et al., 2013). In addition, the ablation of DNMT3a in cultures of mouse embryonic stem cells results in enhanced NPSC proliferation, as well as precocious NPSC differentiation into astrocytes and OLs (Z. Wu et al., 2012).

2.2 OPC Proliferation and Migration

Following OL lineage specification, spinal cord OPCs continue to proliferate as they migrate substantial distances from their sites of origin. The majority of ventral OPCs spread both dorsally and laterally and traverse several levels rostral and caudal to their originating level. In contrast, OPCs that are dorsal in origin migrate to form more than 50% of OLs in the dorsal and lateral regions of the spinal cord (Fogarty et al., 2005). As they migrate, OPCs continually extend their processes, using growth cones at the end of motile filopodia to survey the local environment (Simpson & Armstrong, 1999). They are guided to their final site of myelination by environmental factors such as secreted motogenic molecules, adhesion molecules, and chemotactic cues.

2.2.1 Extrinsic Control

Platelet-derived growth factor is the most important mitogen for maintaining the proliferative state of OPCs in spinal cord WM (Hill, Patel, Medved, Reiss, & Nishiyama, 2013), and is also a key motogenic factor regulating long-range OPC migration (de Castro & Bribián, 2005; Simpson & Armstrong, 1999). The secreted factor activates the PDFGRα receptor on OPCs, stimulating the phosphatidylinositol 3-kinase (PI3K), phospholipase C gamma (PLCγ), and alpha 6 beta 3 (α6β3) integrin signaling pathways (Baron, Shattil, & ffrench-Constant, 2002; McKinnon, Waldron, & Kiel, 2005). Fibroblast growth factor-2 (FGF2) signaling also plays an important role in OPC proliferation (Figure 2.2) and migration by upregulating expression of Olig2 and PDFGRα (Bansal & Pfeiffer, 1997; Fortin, Rom, Sun, Yayon, & Bansal, 2005). Both PDGF and FGF-2 induce rapid OPC division and regulate OPC migration while preventing lineage progression to the immature OL stage (de Castro & Bribián, 2005; Wolswjk & Noble, 1992).

Loss of neural activity in the developing optic nerve (ON), via transection or injection of tetrodotoxin (TTX), suppresses OPC proliferation (Barres & Raff, 1993) and reduces the number of myelinating OLs (Demerens et al., 1996). $NG2^{+}$ OPCs receive synaptic input from axon collaterals in several CNS regions, including the hippocampus, dentate gyrus, corpus callosum, and cerebellum (Bergles, Jabs, & Steinhäuser, 2010). This synaptic input is maintained while OPCs proliferate and migrate, but disappears as they differentiate into immature OLs, suggesting a role for direct synaptic input in the development of

oligodendroglial cells (De Biase, Nishiyama, & Bergles, 2010; Kukley et al., 2008; Kukley, Nishiyama, & Dietrich, 2010). $NG2^+$ OPCs express voltage-gated Na (NaV) channels, along with ionotropic glutamate and GABA receptors, and glutamatergic signaling between neurons and OL lineage cells has been implicated in promoting OL differentiation (De Biase et al., 2010).

2.2.2 Intrinsic Control

The miR-17-92 cluster, along with miRs-9, -214, and -199a-5p and DNMT1, regulates OPC proliferation (Figure 2.2). The gene for the miR-17-92 cluster encodes 12 distinct mature products, 6 of which have been implicated in OPC proliferation: miRs-17, 18a, 19a, 20a, 19b, 92a (Budde et al., 2010; De Faria Jr et al., 2012). This cluster, found in the CNS white matter of humans and rodents (De Faria Jr et al., 2012), is one of most abundant miR families in both OPCs and mature OLs (Budde et al., 2010). microRNA-19b promotes the differentiation of NSPCs into OPCs (Figure 2.1), and keeps OPCs in their proliferative state (Figure 2.2), by inhibiting the tumor suppressor Pten and activating the PI3K/Akt/mTOR pathway (Budde et al., 2010). Oligodendrocyte differentiation is prevented by miR-20a, which suppresses PLP/DM20 levels (Wang & Cambi, 2012). Oligodendrocyte progenitor cells have high levels of miR-9 and miR-214, which increase OPC numbers by targeting serum response factor (SRF), a transcription factor, and the myelin proteins PMP22 and Mobp (Buller et al., 2012; Lau et al., 2008; Letzen et al., 2010). The transcription factor E2F1, expressed in OL lineage cells, binds to UHRF1 and increases DMNT1 activity

and specificity, thereby preventing OPCs from exiting the cell cycle (Bashtrykov, Jankevicius, Jurkowska, Ragozin, & Jeltsch, 2013; Magri et al., 2014).

2.3 OPC Differentiation

An intrinsic “clock”, along with extracellular signals, regulates the fates of proliferating OPCs as they exit the cell cycle and differentiate into post-mitotic, premyelinating OLs. This transition is induced in vitro by mitogen withdrawal and/or the presence of triiodothyronine/thyroid hormone 3 (T3), although T3 is not essential for OPC differentiation (Barres et al., 1994; Gao et al., 1997; Durand and Raff, 2000). Initial OPC differentiation is characterized by the expression of proteolipid protein (PLP), myelin basic protein (MBP), and 2’-3’-cyclic nucleotide 3’-phosphohydrolase (CNP) (Pfeiffer, Warrington, & Bansal, 1993). These immature OLs send out numerous processes to survey the local environment and find the correct axons to myelinate. This stage is transient in rodents, where OLs that fail to make an axonal connection during development undergo programmed cell death (Trapp, Nishiyama, Cheng, & Macklin, 1997). Rodent OLs have ~12 hours to select axons for myelination in vitro, regardless of the final number of sheaths that are made (Czopka, Ffrench-Constant, & Lyons, 2013; Watkins, Emery, Mulinyawe, & Barres, 2008). In contrast, premyelinating OLs in the developing human brain have several months to fine tune their axonal selection (Back, Luo, Borenstein, Volpe, & Kinney, 2002).

2.3.1 Extrinsic Control

Propagating action potentials cause axons to release ATP, which binds directly to astrocytes or is hydrolyzed to adenosine by ectoenzymes. ATP signaling leads to the liberation of leukemia inhibitory factor (LIF) from astrocytes (Cohen & Fields, 2008), which stimulates OL maturation and myelination (Ishibashi et al 2006). Ablation of LIF signaling results in delayed OL differentiation in the developing mouse optic nerve at P10, but the myelination defect recovers by P14, suggesting that LIF is required for the onset, but not the completion, of CNS myelination during development (Ishibashi, Lee, Baba, & Fields, 2009). Adenosine inhibits OPC proliferation, instead promoting differentiation and myelin formation by activating purinergic receptors (Stevens 2002).

2.3.2 Intrinsic Control

microRNAs-219, 138, and 338 are also major regulators of OL differentiation. Enriched in white matter of the adult human brain (De Faria Jr et al., 2012) and postnatal rodent spinal cord (Zhao et al., 2010), these miRs are considered OL-specific because their expression is much higher in OLs relative to both astrocytes and neurons (Dugas et al., 2010). All three are induced by mitogen withdrawal in primary OPC cultures, but not by exposure to T3 (Dugas et al., 2010). Peak expression of miR-219 and miR-338 coincides with the onset of OL maturation in the mouse spinal cord at P20 (Zhao et al., 2010). Together they promote OL differentiation by suppressing the expression of PDGFRα, FGFR2, Sox6, and Hes5, which function to keep OPCs in an undifferentiated state

(Dugas et al., 2010; Zhao et al., 2010). microRNA-219 is the most abundant miR in mature A2B5^{-}GalC^{+} rat OLs (Lau et al., 2008), and it is is a key regulator of rapid OL differentiation that's induced by mitogen withdrawal (Dugas et al., 2010). microRNA-138 is more weakly expressed in rodent CNS white matter and has opposing effects on OL differentiation, promoting the early phase (CNP, MBP) while delaying the later phase (MOG) (Dugas et al., 2010).

Histone deacetylase activity is also required for OL maturation (Figure 2.2). HDAC1/2 downregulate Sox2 expression in OPCs, inducing their differentiation into mature OLs (Shen, Liu, Li, Wolubah, & Casaccia-Bonnefil, 2008). They also outcompete β-catenin for interaction with the transcription factor TCF7L2 during development, and disruption of HDAC1/2 is severely detrimental to OL differentiation (Ye et al., 2009). Sirtuin2 (SIRT2), an OL-specific HDAC, promotes OL differentiation through enhanced MBP expression in the OPC cell line CG4 (S. Ji, Doucette, & Nazarali, 2011). This process is inhibited by Nkx2.2, which recruits HDAC1 to the SIRT2 promoter and negatively regulates its expression. Expression of SIRT2 in the developing CNS is also correlated with that of CNP, where it is found in OL processes and the myelin sheath (Shen & Casaccia-Bonnefil, 2008). Recruitment of HDAC11 to the genes for MBP and PLP correlates with their increased expression in maturing OL-1 cells, a nontransformed rat OL cell line (Hedi Liu, Hu, D'ercole, & Ye, 2009). Valproic acid-induced HDAC inhibition during the first 10 postnatal days impedes OL differentiation in the developing rat corpus callosum, resulting in significant hypomyelination and delayed expression of myelin proteins (Shen et al., 2005).

In contrast, HDAC inhibition does not effect OL maturation or myelination when VPA treatment occurs after the 3rd postnatal week, when differentiation is almost complete, suggesting that HDAC activity is critical for the initial stages of OPC differentiation into postmitotic OLs.

To our knowledge, DNMT-mediated regulation of OL lineage progression has not been established. However, methylation of the Sox10 gene, a TF required for terminal OL differentiation, is correlated with its reduced expression and the repression of other OL-related genes in patients suffering from schizophrenia (Iwamoto et al., 2005), suggesting that DNMT activity may be involved in the regulation of OL development.

2.4 Myelination

Following differentiation and axonal selection, OLs continually extend and retract their processes until a process starts to spiral around an adjacent axon in a cork-screw like manner, resulting in a multiple layers of cell membrane (Ioannidou, Anderson, Strachan, Edgar, & Barnett, 2012). Cytoplasm is extruded and the membrane stack is compacted to form functional myelin. Each OL can generate up to 50 internodes during development (Pfeiffer et al., 1993). However, the number and length of internodes depends on the animal's age at the time of myelin synthesis (Young et al., 2013).

2.4.1 Extrinsic Control

Neural activity induces the vesicular release of glutamate from axons (Wake, Lee, & Fields, 2011), which triggers signaling cascades by activating NMDA and AMPA receptors on oligodendroglial cells. While both receptors are responsible for activity-dependent inward Ca^{2+} currents, they play different roles in the oligodendrogenic developmental program (De Biase et al., 2011). AMPA receptor (AMPAR) signaling activates the Fyn kinase pathway, enhancing local MBP synthesis in OLs (Wake et al., 2011). Instead of regulating OL development, NMDA receptor (NMDAR) signaling is used to control the expression of Ca^{2+}-permeable AMPA receptors on OL lineage cells. OPCs lacking the NMDAR subunit R1, which is required for normal NMDAR signaling, are able to proliferate, differentiate, myelinate major WM tracts, and form glutamatergic synapses normally (De Biase et al., 2011).

2.4.2 Intrinsic Control

Known epigenetic regulators of terminal OL maturation and myelin protein synthesis includes miR-219, miR-23a, miR-214, and the miR-17-92 cluster. microRNA-219 influences lipid accumulation by inhibiting the protein ELOLV7 (Shin, Shin, McManus, Ptácek, & Fu, 2009), whereas miR-20a of the miR-17-92 cluster inhibits myelination by suppressing expression of the myelin proteins PLP/DM20 (Wang & Cambi, 2012). Using transgenic mice that express miR-23a under control of the CNP promoter, Lin et al. (2013) observed that miR-23a overexpression stimulates hypermyelination in the corpus callosum by increasing

myelin thickness and promoting proper myelin folding. Excess miR-23a induces both OPC differentiation (MBP, PLP) and myelin synthesis (MAG, MOG) through two separate mechanisms: suppression of lamin B1 levels and activation of the Akt/mTOR pathway (S.-T. Lin & Fu, 2009; S. Lin et al., 2013). Demethylation of the MAG gene is associated with OL maturation and myelin synthesis in vitro (Grubinska, Laszkiewicz, Royland, Wiggins, & Konat, 1994). Similar demethylation patterns may occur with other coordinately expressed myelin-specific genes but they have yet to be identified.

Figure 2.1. Epigenetic regulation of CNS cell lineage specification. Epigenetic factors such as miRs, HDACs, and DNMTs influence signaling pathways in NSPCs, resulting in the inhibition (red) or promotion (green) of astrocyte, neuron, or OL lineage specification.

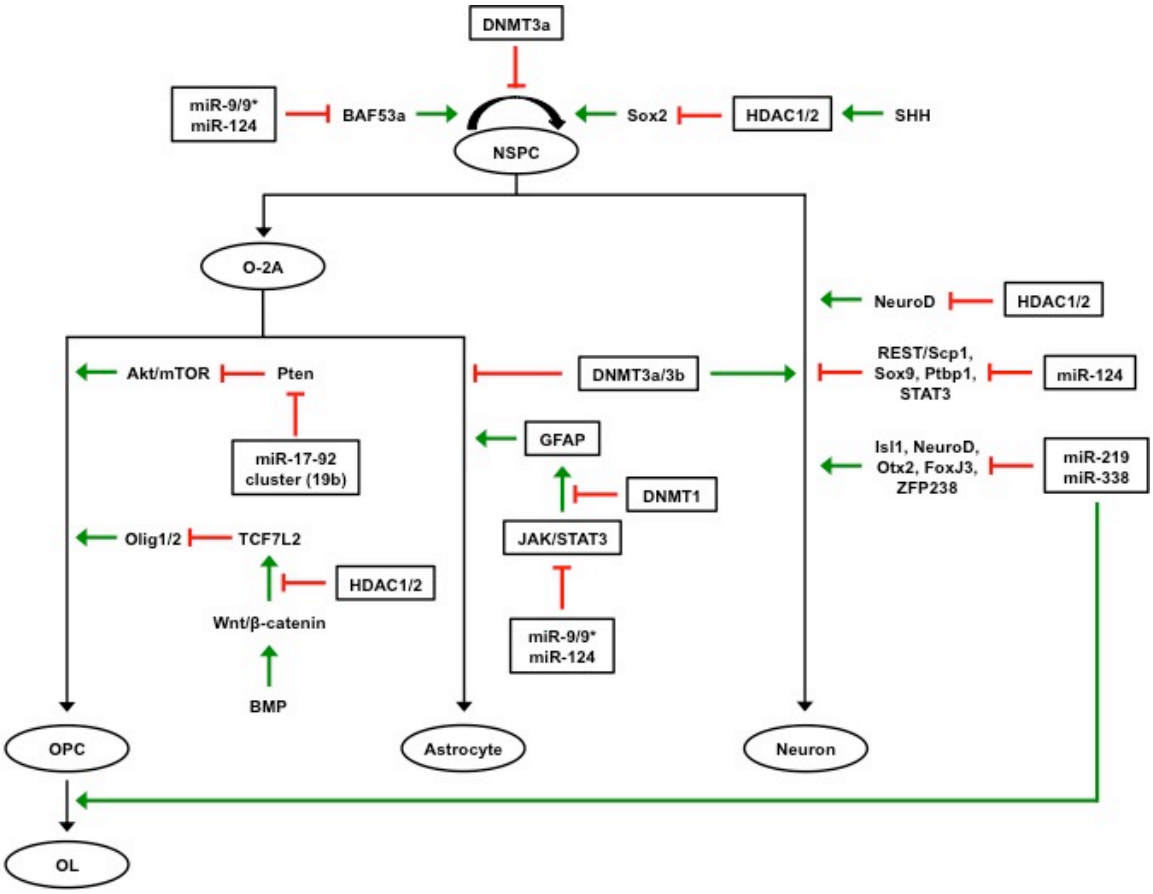
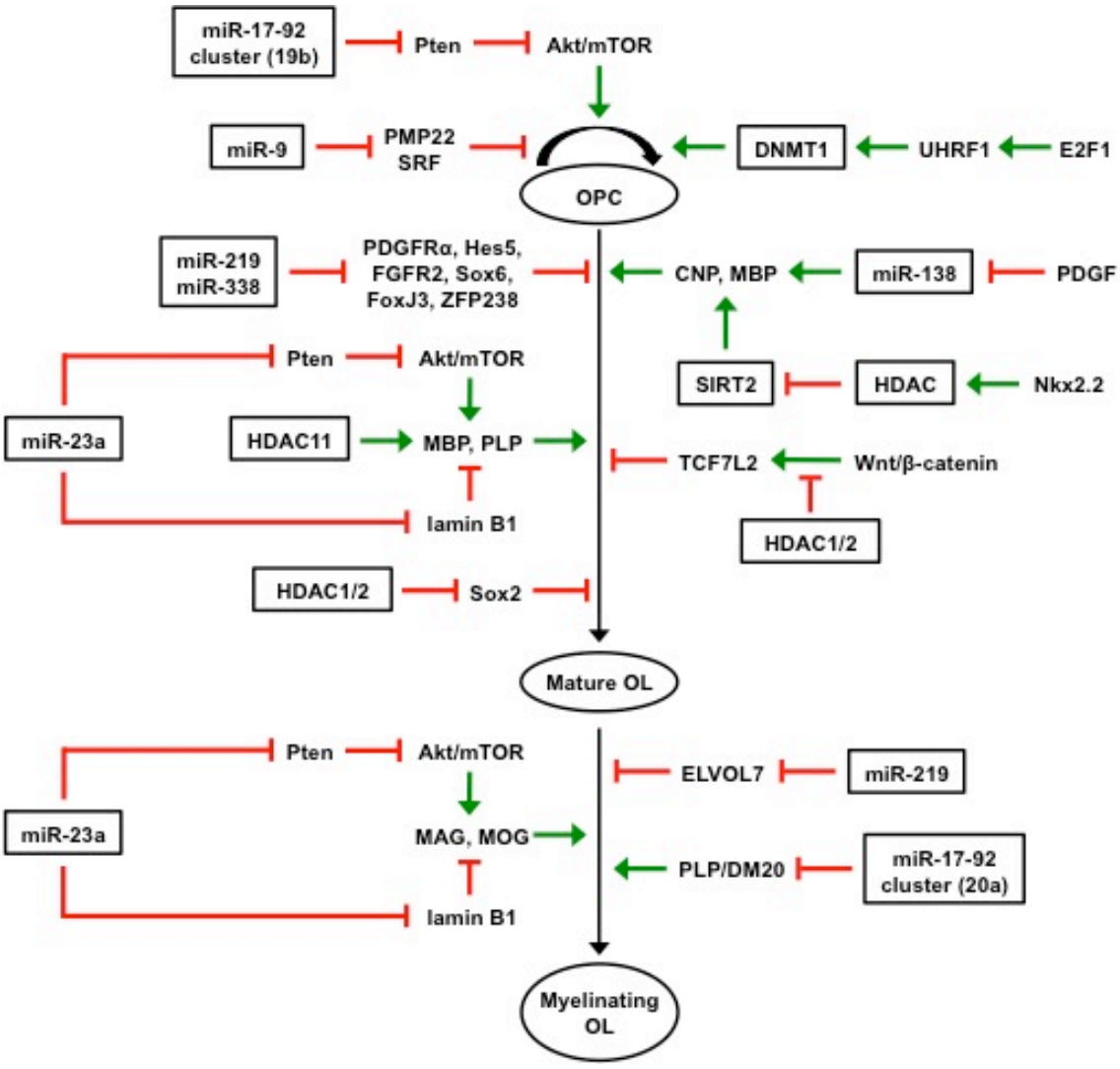


Figure 2.2. Epigenetic control of OL lineage progression.

Epigenetic factors inhibit (red) or promote (green) expression of their target genes in every phase of oligodendroglial proliferation, differentiation, and myelination.



Chapter 3: Functional Electrical Stimulation after Chronic Spinal Cord Injury in Adult Rats

3.1 Chapter Overview

Our lab has developed a rodent model of functional electrical stimulation (FES) that alternately stimulates the large myelinated fibers of the common peroneal nerves in the tibialis anterior muscles. This model produces a crude approximation of hind limb bilateral stepping and, thereby, returns patterned neural activity that is typically diminished below the lesion level after a spinal cord injury (SCI). Using this model, Becker et al. found that applying FES after a chronic thoracic transection injury in adult rats enhances the proliferative response of neural precursor cells in a region of the lumbar spinal cord (Becker et al., 2010) that is associated with the central pattern generator (CPG) for locomotion (Edgerton et al., 2001). However, that study only utilized a single frequency (20 Hz), and several studies have shown that the extent of oligodendrocyte (OL) survival, myelination, and axonal regeneration after electrical stimulation depends on the stimulation frequency (Gary et al., 2012; Malone et al., 2013; Udina et al., 2008). The purpose of this study was to

determine if FES treatment influences the proliferation of cells in the OL lineage after a chronic complete thoracic SCI in adult rats, and if these changes are frequency dependent.

3.2 Methods

3.2.1 Experimental Design

Adult female Long Evans rats (200-250 g upon arrival; Harlan) received a complete transection at vertebral level T9. FES treatment began 1 month post-injury (PI), and continued for 3 weeks (Figure 3.2). Bromodeoxyuridine (BrdU) (50 mg/kg i.p.), a thymidine analogue that is incorporated into replicating DNA, was injected daily during the last 5 days of stimulation to identify dividing cells. The experimental groups (n = 10 per group) were:

1) Control, no FES
2) FES at 2 Hz
3) FES at 20 Hz
4) FES at 100 Hz

3.2.2 Animal Care

Animals were anesthetized with an intraperitoneal (IP) injection of dexdomitor (0.4 mg/kg; Pfizer Animal Health 6295) and ketathesia (60 mg/kg; Butler Schein Animal Health 043012). Controlled heating pads maintained animal body

temperature during surgery and the recovery period. Anesthesia was reversed with an intramuscular injection (IM) of 0.05 ml antisedan (1 mg/kg; Pfizer Animal Health 6298). Daily injections of Lactated Ringer's solution (10 ml; Henry Schein 1002435) and 0.03 ml of gentamycin (40 mg/ml; Henry Schein 1049944) were given subcutaneously and IM, respectively, for up to a week after surgery. Bladders were expressed twice daily for the duration of the experiment.

3.2.3 Spinal Cord Injury

A midline incision was made in the upper back, and the overlying musculature was dissected to reveal the dorsal and lateral processes of the spinal column. A dorsal laminectomy at vertebral level T9 exposed the spinal cord, and a small slit was made in the dura using a 20G needle (Becton Dickinson 305175). A metal canula, attached to a vacuum pump (Bio-rad), applied 20 in Hg of suction to remove approximately 3 mm of spinal cord tissue through the dural slit, thereby completely transecting the spinal cord. This suction ablation injury model minimizes damage to the dura while eliminating all ascending and descending neural activity across the lesion, allowing us to determine both the local and global effects of FES. After the injury, the overlying musculature was sutured together and the skin incision closed with surgical staples. Animals were allowed to recover overnight in heated cages, with food and water *ad libitum*, and then housed in unheated cages for the duration of the experiment.

3.2.4 Electrode Implantation

The FES implant (Figure 3.1) consisted of a plastic pedestal connected to 3 stainless steel electrode pads (1 mm x 0.6 mm x 3.18 mm) with nylon-coated stainless steel wires (PlasticsOne MS333/76H). The pedestal was secured in a harness (Instech CIH95AB) around the animal's upper torso, while an incision in the skin of the upper back allowed the electrode leads to be tunneled subcutaneously. A stimulating electrode pad was sutured into the tibialis anterior muscle of each hind limb, adjacent to the common peroneal nerve, and the ground electrode was sutured to the musculature of the lower back near the midline. All animals were fitted with plastic collars after electrode implantation to prevent autophagia and housed in separate cages. FES treatment began 3 days after electrode implantation.

3.2.5 Functional Electrical Stimulation

A two-channel swivel commutator (PlasticsOne SL2C) was secured to the inside of each cage lid and connected to the electrode pedestal with a spring-covered bipolar cable (PlasticsOne) during FES. An external stimulator was connected to the commutators to provide stimulation. Animals were disconnected from the commutators daily after the cessation of treatment. Action potentials were induced using biphasic pulses (3 mA, 200 μs) for 1 hour, 3 times per day, at varying frequencies. The stimulation pattern was: 1 s stimulation of one common peroneal nerve, then 1 s of rest, followed by 1 s stimulation of the other common peroneal nerve, and 1 s of rest. These stimulation parameters preferentially

activate the large myelinated fibers of the common peroneal nerve (Gorman & Mortimer, 1983; Grill & Mortimer, 1996), which is expected to increase afferent activity in the lumbar spinal cord (Rivero-Melián, 1996; Swett & Woolf, 1985). All animals underwent SCI and electrode implantation, but control animals did not receive FES treatment.

3.2.6 Tissue Processing and Immunohistochemistry

Seven weeks after injury, animals were anesthetized as described above and perfused transcardially with 300 mL of saline followed by 500 mL of ice-cold 4% paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS). Spinal cords were removed and post-fixed overnight in PFA at 4°C, then cryoprotected with 30% sucrose and 0.2% sodium azide in PBS. Serial transverse sections (40 μm) were cut in a 6-series from spinal cord segments rostral (T2/T3) and caudal (L2/L3) to the lesion and stored in PBS.

Sections were fixed in absolute methanol on ice for 10 minutes, washed with PBS, and incubated in 2M HCl for 60 min at 37°C. A twenty-minute rinse with 0.1M borate buffer was used to neutralize the acid. Following a PBS wash, the tissue was incubated in 5% normal goat serum (NGS), 5% normal donkey serum (NDS), and 1% bovine serum albumin (BSA) in 0.4% triton X-100/PBS at room temperature for 1 hour to permeabilize the tissue and block non-specific labeling. Treatment with primary antibodies occurred over two nights at 4°C in diluted blocking solution (1:3 in PBS). The primary antibodies used were: rat anti-BrdU (1:150; Serotec MCA2060), rabbit anti-NG2 (1:250; Millipore AB5320), and

mouse anti-APC-CC1 (1:125; Calbiochem OP80). The tissue was then incubated with secondary antibodies diluted in PBS for 2 hours at room temperature. The secondary antibodies used were: donkey anti-rat Alexa 549 (1:100; Jackson Immuno Research 712-505-153), and goat anti-rabbit (1:250; Molecular Probes A-11034) or anti-mouse (1:200; Molecular Probes A-11029) Alexa 488.

3.2.7 Cell Quantification

A stereology system (Stereoinvestigator, MBF Bioscience) was used to quantify the total number of dividing cells ($BrdU^+$), oligodendrocyte progenitor cells (OPCs) ($BrdU^+/NG2^+$) and mature OLs ($BrdU^+/APC^+$) in the entire white matter. Tissue sections were outlined at low magnification (4x) to specify the sampling area, and quantification was performed at 60x. The optical fractionator superimposed an unbiased grid (Figure 3.2) on the cross sections (thoracic: 375 µm x 275 µm; lumbar: 300 µm x 450 µm). The counting frame (80 µm x 80 µm), dissector height (12 µm), and guard zone distance (3 µm) were identical at all levels sampled. Grid and counting frame dimensions were chosen so that quantification resulted in a coefficient of error less than or equal to 15%. Ten sections per level were used for quantification. Animals were excluded from statistical analysis if their $BrdU^+$ cell counts were greater than 2 standard deviations from the mean. Comparison between experimental groups and spinal cord levels was performed using a two- way ANOVA and Bonferroni post-test

(Graphpad Prism). Statistical significance was accepted at $p < 0.05$, and all data are presented as mean ± SEM.

3.3 Results

3.3.1 FES Promotion of Cell Division is Frequency-dependent

Previous work from our lab has shown that FES at 20 Hz promotes overall cell proliferation at L1 and L5 after a complete mid-thoracic spinal cord complete transection (Becker et al., 2010). We sought to determine if this increase is dependent on the stimulation frequency. Following a complete mid-thoracic transection (T9), adult rats were allowed to recover for 4 weeks, then received 3 weeks of daily FES treatment at 2, 20, or 100 Hz (Figure 3.2). Control animals not receive stimulation. Daily injections of BrdU were delivered for 5 days prior to sacrifice to label dividing cells, and these cells were quantified in the entire white matter (WM) rostral and caudal to the injury level. $BrdU^+$ nuclei were distributed throughout the WM at both levels (Figure 3.3), but rarely detected in the gray matter (GM). The highest concentration occurred in areas where the dorsal and lateral ascending sensory tracts are located. At vertebral level T2/T3, BrdU incorporation was most evident in the dorsal columns and where lateral ascending sensory tracts are located. Cell proliferation caudal to the lesion was also observed in the sensory tracts of the lateral and ventral WM, while lacking in the dorsal columns.

The number of $BrdU^+$ cells rostral to the lesion was not influenced by FES treatment, regardless of stimulation frequency (Figure 3.2). Complete spinal cord transection did not depress the number of dividing cells caudal to the lesion, in contrast to previous findings (Becker et al., 2010). Animals that received stimulation at 20 Hz (36.07 ± 2.0; $p < 0.01$) and 100 Hz (34.05 ± 2.99; $p < 0.05$) had significantly enhanced cell proliferation relative to the control group (26.21 ± 1.45) in the lumbar spinal cord, while animals in the 2 Hz group (33.51 ± 3.28) did not. These results indicate that FES of the lower limbs modulates cell division in a frequency-dependent manner, and these changes are restricted to lumbar spinal cord.

3.3.2 FES at 20 Hz Enhances OPC Proliferation

Functional electrical stimulation is known to increase the number of neural progenitor cells in the lumbar spinal cord after a mid-thoracic transection (Becker et al., 2010). To determine if FES frequency influences how many of these cells enter the OL lineage, we assessed the number of $BrdU^+$ cells that became OPCs based on co-labeling with NG2. OPC proliferation as a function of FES treatment or frequency remained unchanged above the injury level (Figure 3.4). Conversely, FES at 20 Hz augmented the number of $BrdU^+/NG2^+$ cells in the lumbar spinal cord to 13,831.87 ± 964.58, over a 50% increase relative to control animals (9,002.29 ± 1,043.39; $p < 0.05$) and those that received treatment at 2 Hz (9,278.5 ± 894.32; $p < 0.05$). Stimulation did not affect the number of $BrdU^+$ cells that were co-labeled with NG2 between spinal cord levels. These

observations suggest that 20 Hz is the optimal FES frequency to use in adult rats in order to maximize OPC proliferation in the lumbar spinal cord.

3.3.3 Increased Proportion of Newborn OLs Between Spinal Cord Levels After FES Treatment

Oligodendrocyte differentiation and myelination can be enhanced by electrical stimulation in vivo and in vitro (Impey et al., 2010; Q. Li et al., 2010; Malone et al., 2013). We quantified the number of BrdU+ cells that also expressed APC in the injured spinal cord to assess if FES treatment at varying frequencies can augment OPC differentiation into mature OLs. Stimulation did not impact the number of $BrdU^+$ cells that were co-labeled with APC between experimental groups or spinal cord levels (Figure 3.5). However, a greater proportion of these cells were observed caudally (40.34% ± 3.38) versus rostrally (26.5% ± 2.25) in animals that received stimulation at 20 Hz (data not shown; $p < 0.05$). These findings indicate that FES at 20 Hz can increase the number of mature OLs in the lumbar enlargement after a chronic mid-thoracic spinal cord injury.

Figure 3.1. Experimental design.

Schematics illustrating the (A) experimental timeline, (B) FES electrode, and (C) electrode placement in adult rats. (D) Schematic showing the stereological quantification of cells in the entire white matter of transverse spinal cord sections (40 μm). Grid and counting frame dimensions are not drawn to scale.

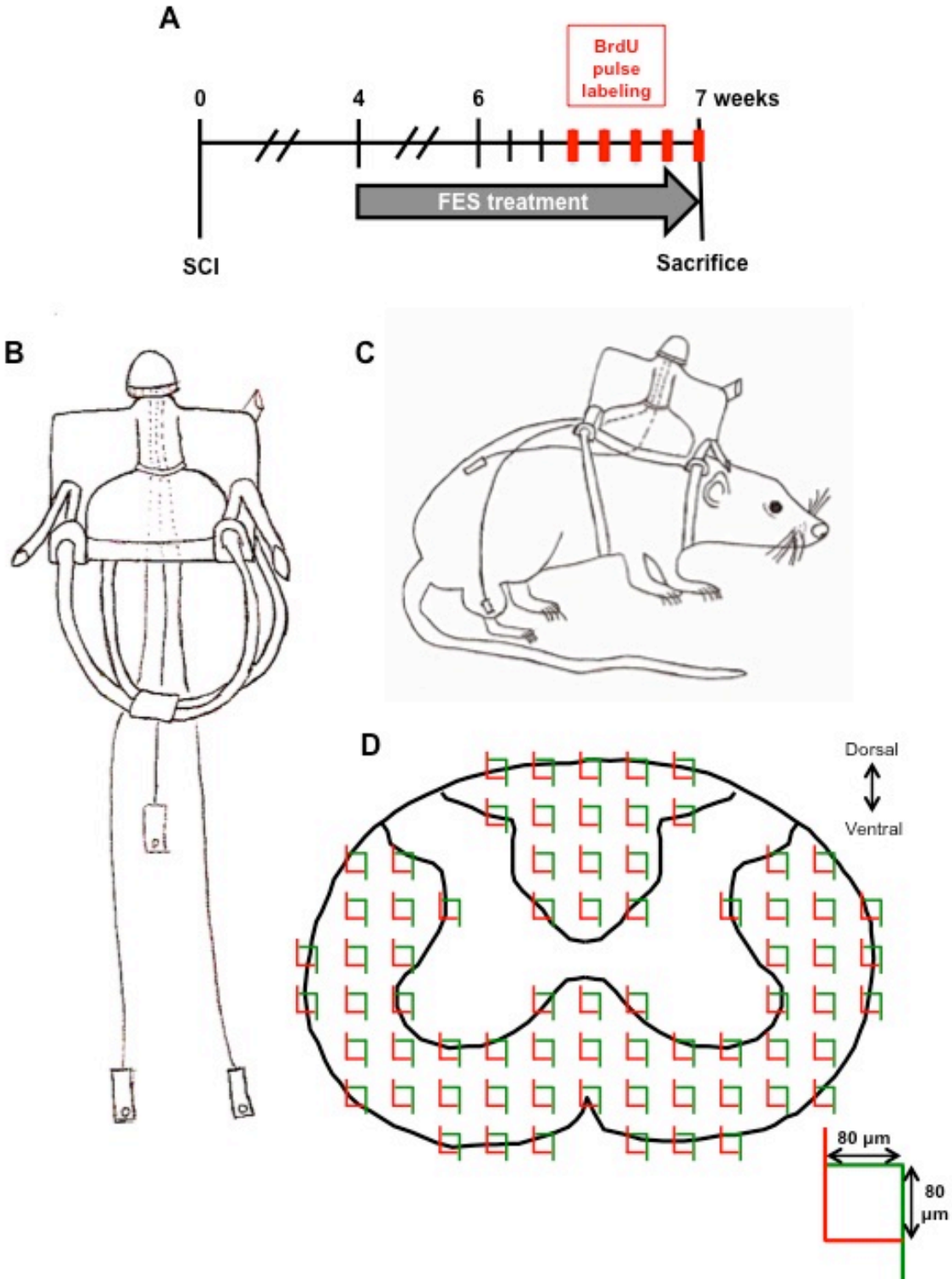

A
BrdU pulse labeling
0
4
6
7 weeks
FES treatment
SCI
Sacrifice
B
C
D
Dorsal
Ventral
80 μm
80 μm

Figure 3.2. FES increases overall cell division.

(A – B) Distribution of BrdU+ cells (red) in transverse spinal cord sections (A) rostral and (B) caudal to the spinal cord lesion. (C) Animals that received FES treatment at 20 ($p < 0.01$) and 100 Hz ($p < 0.05$) had increased cell proliferation in the lumbar spinal cord relative to control animals. Values displayed as mean ± SEM. (control: 26.21 ± 1.45; 2 Hz: 33.51 ± 3.28; 20 Hz: 36.07 ± 2.00; 100 Hz: 34.05 ± 2.99).

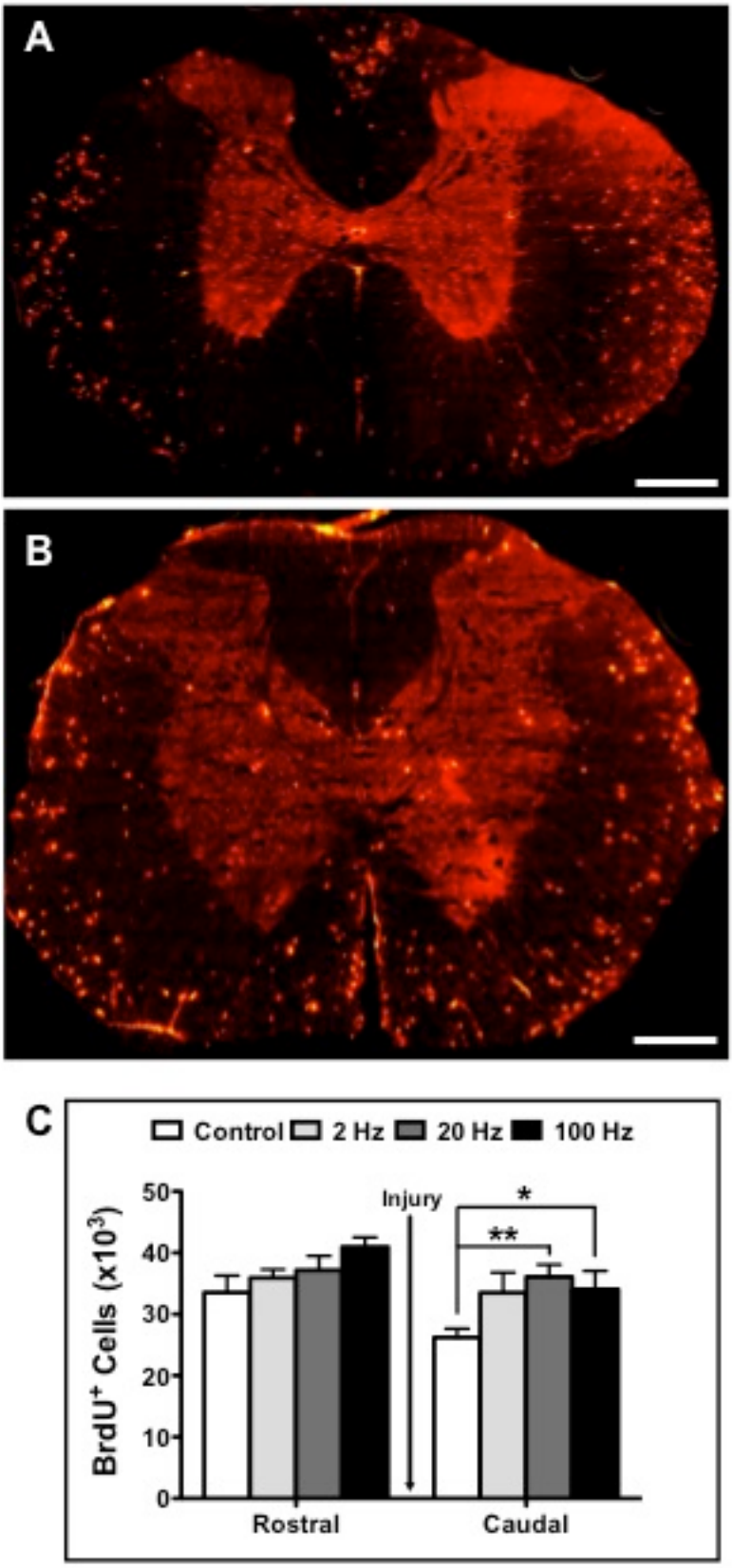

A
B
C
Control
2 Hz
20 Hz
100 Hz
BrdU+ Cells (x10^3)
50
40
30
20
10
0
Injury
*
**
Rostral
Caudal

Figure 3.3. FES enhances OPC proliferation.

(A) FES at 20 Hz increases the number of proliferating OPCs relative to control animals ($p < 0.05$) and those that received treatment at 2 Hz ($p < 0.05$) caudal to the SCI. Values displayed as mean ± SEM. (control: 9.0 ± 1.04, 2 Hz: 9.28 ± 0.89, 20 Hz: 13.83 ± 0.96, 100 Hz: 12.19 ± 1.39). (B) Schematic illustrating the location of the images C – G in transverse spinal cord sections. (C – D) Representative images of the $NG2^+$ cell distribution in the (C) dorsal columns and (D) ventral white matter caudal to the lesion. (E – G) High-magnification images show the morphology of $BrdU^+/NG2^+$ cells.

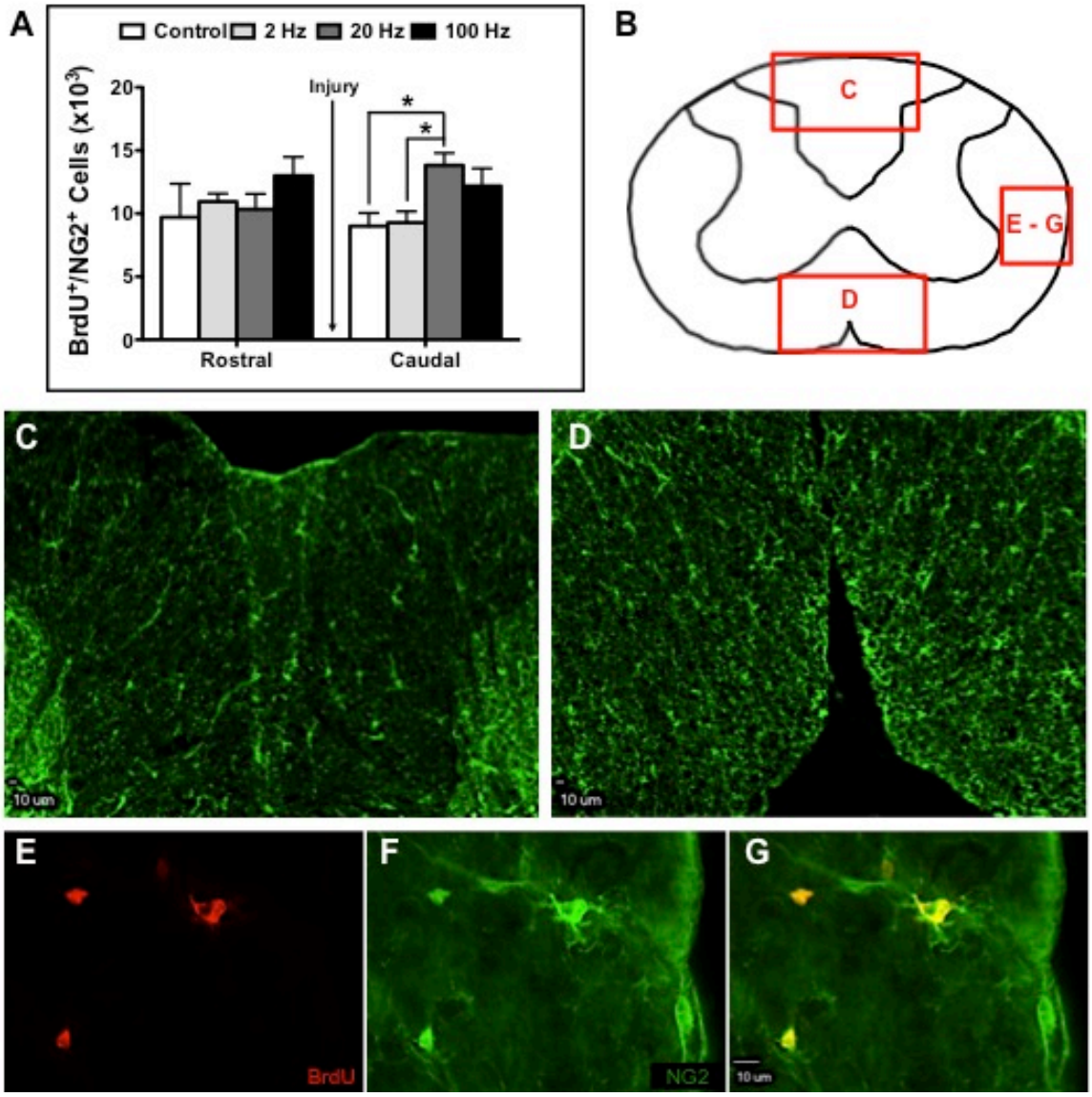
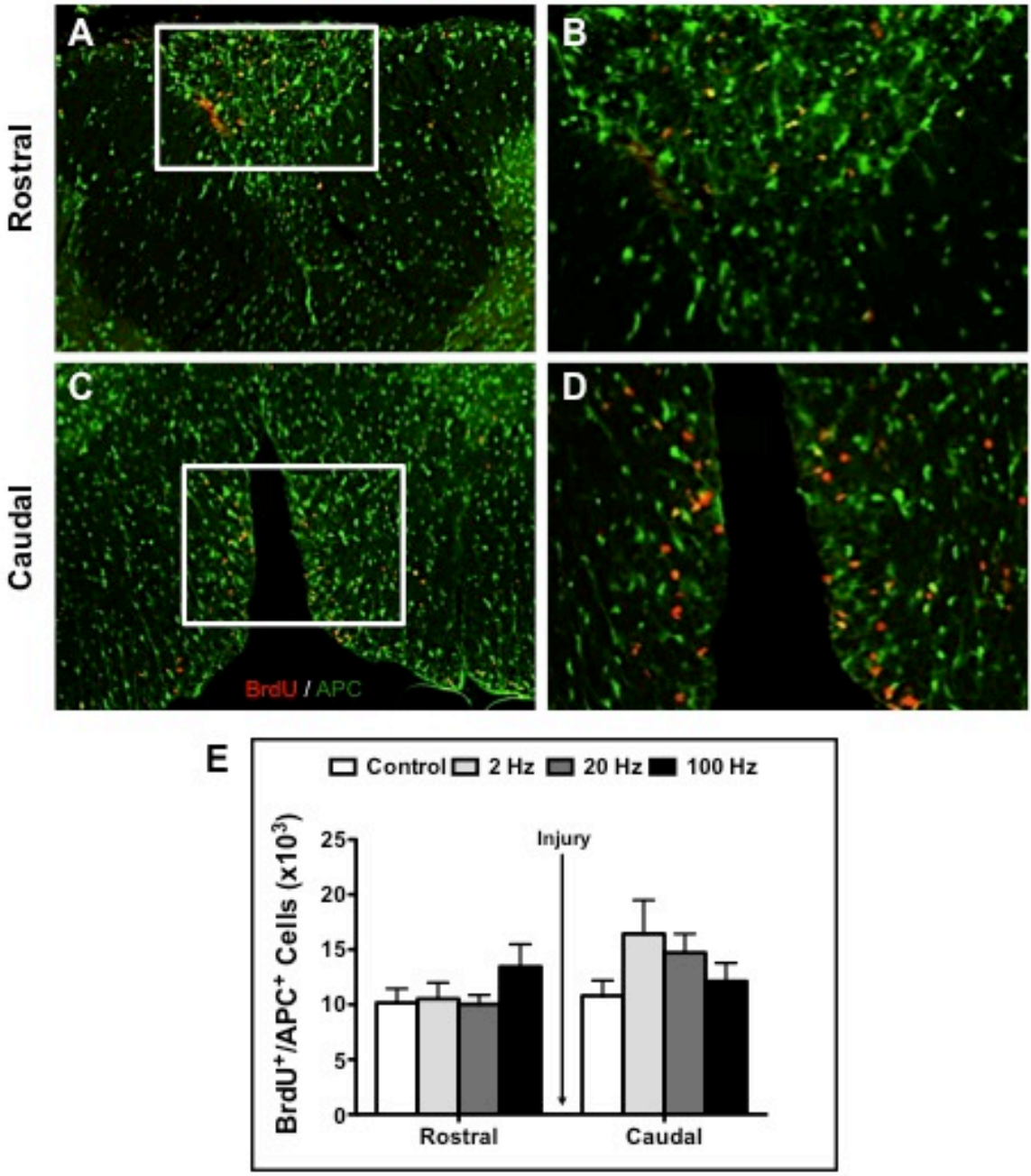

A
Control
2 Hz
20 Hz
100 Hz
BrdU+/NG2+ Cells (x10^3)
20
15
10
5
0
Injury
*
*
Rostral
Caudal
B
C
E - G
D
C
10 um
D
10 um
E
BrdU
F
NG2
G
10 um

Figure 3.4. FES does not influence the number of mature OLs.

(A – D) Representative images of $BrdU^+/APC^+$ cells in the (A, B) dorsal columns and (C, D) lateral WM. (B, D) High-magnification images from boxed areas in (A) and (C). (E) The number of $BrdU^+$ OLs was not affected by treatment group or spinal cord level. Values displayed as mean ± SEM.



Chapter 4: Short-term Functional Electrical Stimulation after Acute Spinal Cord Injury

4.1 Chapter Overview

Short-term electrical stimulation of nervous tissue can augment activity-dependent mechanisms such as axonal regeneration and remyelination both in vivo and in vitro (Udina et al., 2008; Wan et al., 2010). However, the effect of short-term ESTIM on oligodendrocyte (OL) development in vivo has not been fully investigated. The purpose of this study was to assess if short-term FES treatment can enhance the development of adult oligodendrocyte progenitor cells (OPCs) harvested from the injured adult rat spinal cord. Using immunopanning to isolate OPCs from rostral and caudal to a complete transection injury, we determined that one day of FES treatment at 20 Hz, given overnight prior to cell isolation, promotes OPC differentiation without influencing OPC proliferation or process elongation.

4.2 Methods

4.2.1 Experimental Design

Adult, female Long Evans rats (200-250g upon arrival; Harlan) were injured at spinal cord level T9 and implanted with electrodes for FES as described above. After recovering for three days, animals received three one-hour sessions of FES treatment at 20 Hz overnight. This frequency was chosen based on our previous finding that 20 Hz stimulation increases OPC proliferation in the injured adult spinal cord. Control animals did not receive stimulation. Oligodendrocyte progenitors were purified from injured adult rat spinal cords by sequential immunopanning and cultured as described below.

4.2.1.1 Primary Cell Culture

Oligodendrocyte progenitors were purified from the injured spinal cord of adult Long Evans rats by sequential immunopanning. Two tissue segments (~15 mm long) equidistant from the injury site were excised from the spinal cord (Figure 4.1). Each segment was digested enzymatically with collagenase and papain (20 units/ml), then passed through a 40-μm filter to remove cell aggregates. The resulting cell suspension was incubated on an untreated Petri dish for 30 minutes at 37°C to remove microglia. Non-adherent cells were then incubated Petri dishes coated with an anti-PDGFRα antibody (Abcam AB61219) for 1 hour at 37°C to select the OPCs. The purified cells were incubated with 0.05% trypsin for ~5 minutes and detached from the plate using a disposable cell scraper.

OPCs were plated at 500 cells/µl in 96-well plates coated with 10 µg/ml poly-D-lysin (PDL) in differentiation or proliferation media.

4.2.1.2 Media Components

Differentiation Media: DMEM media supplemented with 0.5% fetal bovine serum (FBS; Hyclone), 1% glutamax, 1% sodium pyruvate, 1% nonessential amino acids, 8% n-acteyl cysteine, and a N1/N2 supplement (Sigma/Invitrogen). Mitogen withdrawal was used to induce differentiation.

Proliferation Media: Differentiation media was supplemented with fibroblast growth factor (FGF, 10 ng/ml; Invitrogen), neurotrophin-3 (NT-3, 5 ng/ml; R&D Systems), a N1/N2 supplement, and platelet derived growth factor (PDGF, 10ng/ml; R&D Systems) to keep OPCs in an undifferentiated state.

4.2.2 Immunocytochemistry

Cultures were fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature or 30 minutes at 4°C. Cells were blocked with 10% NGS and 0.1% triton in phosphate-buffered saline (PBS) for 1 hour at room temperature. Treatment with primary antibodies occurred over 2 hours at room temperature in the blocking solution. Primary antibodies included rabbit anti-NG2 (1:250; Millipore), mouse anti-myelin basic protein (MBP, 1:1000; Calbiochem), mouse anti-BrdU (1:1000, Sigma), mouse anti-O4 (1:50), and mouse anti-O1 (1:50). Then the cultures were incubated with secondary antibodies goat anti-rabbit Cy3 (1:250; Jackson Immmuno), goat anti-mouse Cy3 (1:250; Jackson Immmuno), or

goat anti-mouse IgM Alexa 488 (1:500; Invitrogen) for 1 hour at room temperature in PBS. Nuclei were stained with Hoescht 33342 (1:2000; Molecular Probes).

4.2.3 Cell Quantification

Cells were counted by scanning the entirety of each well at 20x. Experimental groups and spinal cord levels were compared using a two-way ANOVA and Bonferroni post-test (Graphpad Prism). Significance was accepted at $p < 0.05$. All data is presented as the mean ± SEM.

4.3 Results

4.3.1 Short-term FES Does Not Modulate OPC Proliferation *In Vitro*

Neural activity is required for OPC proliferation in CNS development (Barres & Raff, 1993), and can be used to increase the number of dividing progenitor cells in both the intact (Q. Li et al., 2010) and injured (Becker et al., 2010) spinal cord of adult rats. In our first experiment, we wanted to determine if short-term stimulation could have a similar effect after an acute SCI. Isolated OPCs were cultured for 3 days in proliferation media supplemented with BrdU to label dividing cells and fixed after 3 DIV. Quantification of $BrdU^+$ cells showed that

short-term FES does not modulate OPC proliferation in vitro regardless of the spinal level they originated from (Figure 4.1).

4.3.2 OL Mean Elliptical Area is Not Influenced by FES Treatment

As oligodendrocytes mature and begin myelination, they extend their cellular processes to locate and ensheath nearby axons. We investigated the effect of FES on process extension by measuring the mean elliptical area of $O1^+$ mature OLs after 7 DIV in differentiation media (Figure 4.1). Mean elliptical areas did not differ between experimental groups or spinal cord levels, suggesting that OL process extension is not influenced by short-term FES.

4.3.3 FES Enhances Differentiation of Harvested OPCs

Next we wanted to determine if short-term FES influences OPC differentiation. Therefore, we cultured the harvested OPCs in mitogen-free differentiation media and fixed the cells after 1 or 7 days in vitro (DIV). Antibodies against NG2 and O4 were used to label OPCs and immature OLs, while antibodies against O1 and MBP were used to identify mature OLs. The percentage of cells expressing NG2 or O4 did not change as a function of time in culture, FES treatment, or spinal cord level (Figure 4.2). In control cultures, 50% of cells from above the injury were $O1^+$ or MBP^+ at 7 DIV. In contrast, 12.37% ± 3.73 of cells were labeled with O1 ($p < 0.05$) and 24.58% ± 4.59 were labeled with MBP ($p < 0.05$), decreases of 76% and 52% respectively. Short-term FES at 20 Hz reversed the trend, elevating the number of $O1^+$ and MBP^+ to 56.4% ± 10.4 ($p < 0.05$; versus control)

and 64.87% ± 6.62 ($p < 0.01$; versus control). These findings indicate that short-term stimulation can enhance differentiation of adult OPCs isolated from the injured spinal cord into mature OLs.

Figure 4.1: FES does not influence oligodendroglial proliferation or process elongation.

(A) Schematic of SCI and OPC harvest sites. Short-term FES does not influence (B) OPC proliferation or (C) process elongation of mature OLs. Values displayed as mean ± SEM. (D) Schematic of OL process extension quantification.

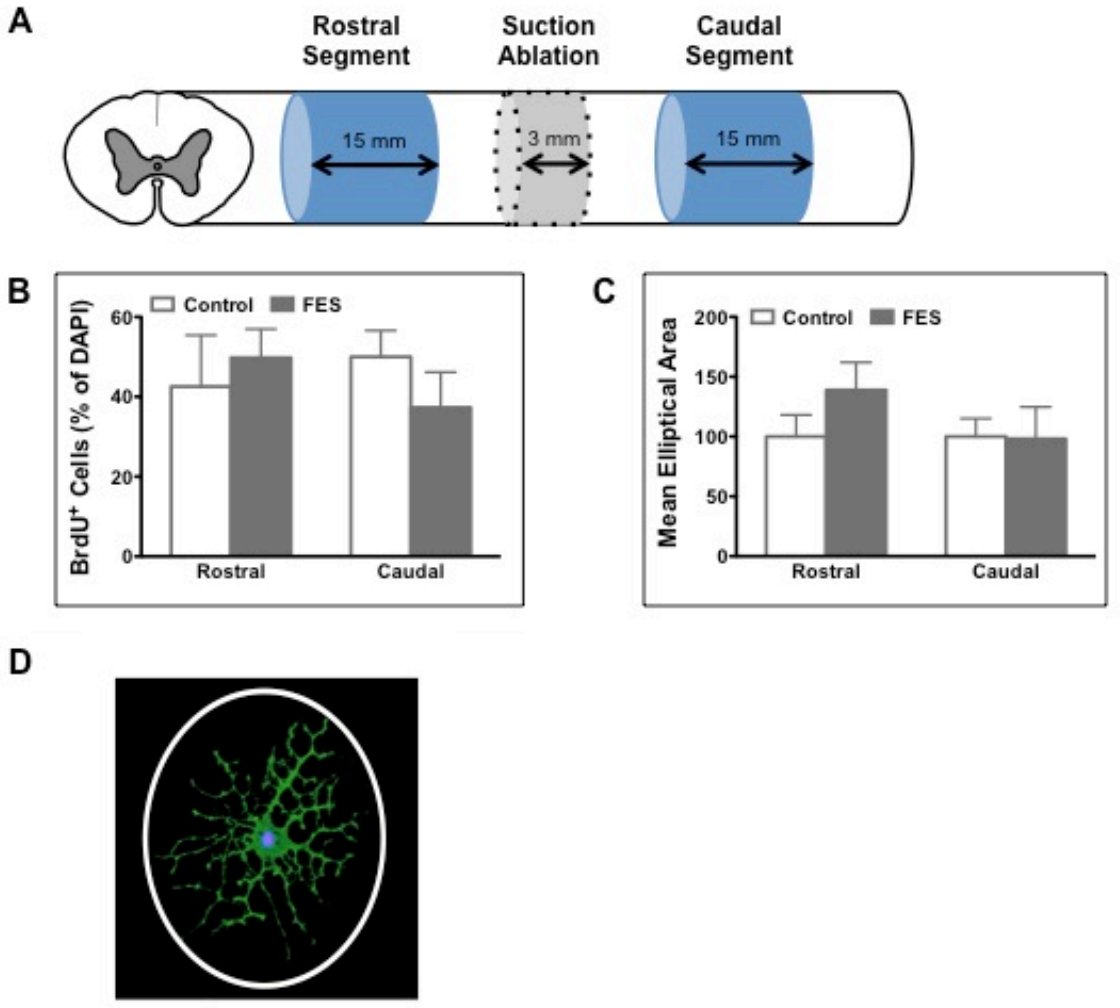
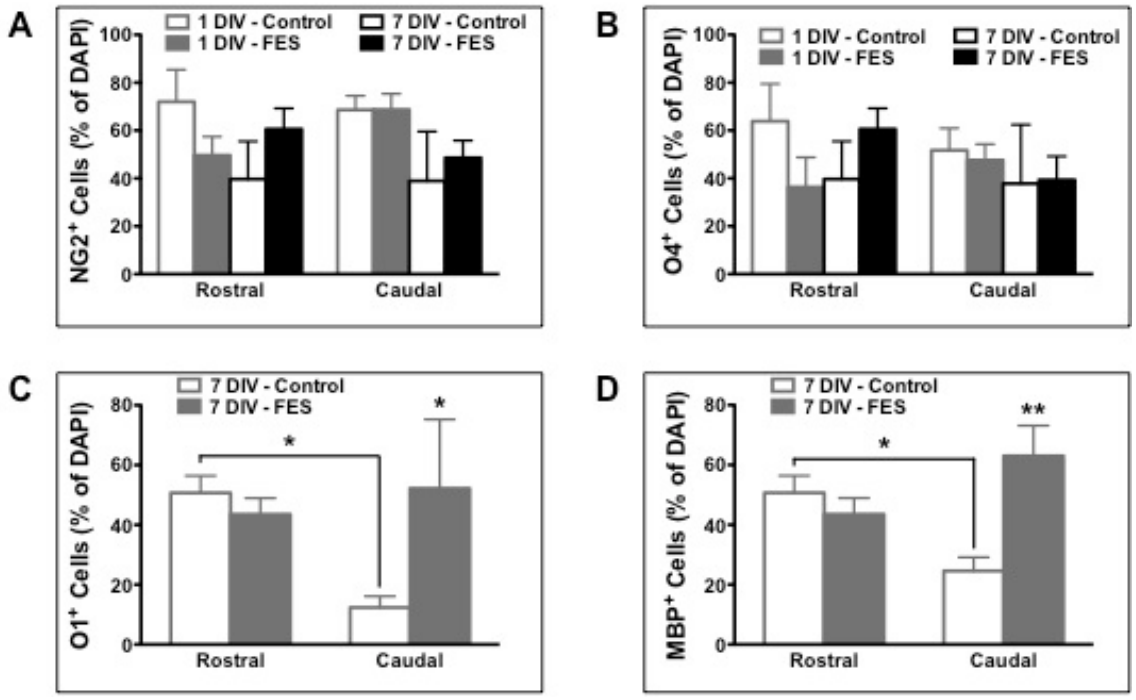


Figure 4.2. FES increased OPC differentiation into mature OLs.

Quantification of OPCs harvested from rostral and caudal to the lesion level after short-term FES and cultured in differentiation media for up to 7 days. Cells were stained with antibodies against OL makers: (A) NG2, (B) O4, (C) O1, and (D) MBP to determine lineage progression. Values displayed as mean ± SEM. P-values determined using two-way ANOVA followed by the Bonferoni post-test. *p < 0.05, **p < 0.01.



Chapter 5: Neural Activity Influences Oligodendrocyte Development by Modulating Epigenetic Mechanisms

5.1 Chapter Overview

Neural activity and epigenetic mechanisms, such as microRNAs (miRs) and chromtin remodeling by histone deacetylases (HDACs) and DNA methyl transferases (DNMTs), have been implicated in the control of oligodendrocyte (OL) development (Zuchero & Barres, 2013). However, the interaction between these regulators of the oligodendrogenic program has yet to be determined. We investigated the dynamics of these epigenetic modulators in OLs after electrical stimulation (ESTIM) in vitro, and in the injured rat spinal cord after functional electrical stimulation (FES) treatment. Using quantitative real-time polymerase chain reaction (qRT-PCR), we determined that ESTIM alters the expression of the miR-17-92 cluster and miR-338 in co-cultures of primary spinal cord OPCs and dorsal root ganglia (DRG). We found that a mid-thoracic complete transection results in miR dysregulation in the lumbar enlargement up to 2 weeks post-injury (PI), but FES at 20 Hz can delay the inhibition of miRs-138 and 338

while simultaneously upregulating the expression of miR-19b, DNMT1, and DNMT3b.

5.2 Methods

5.2.1 Primary Cell Culture and ESTIM

Primary DRG and spinal cord OPCs were harvested from Sprague Dawley rats at postnatal day 4-5 (P4-5). Dorsal root ganglia were allowed to mature in culture for 2 weeks, then harvested spinal cord OPCs were added to the culture (Figure 5.1). One day after plating, co-cultures received one hour of electrical stimulation (ESTIM) at 20 HZ daily for up to 7 days in vitro (DIV). Cells were fixed or harvested for RNA isolation immediately after the last ESTIM session.

5.2.1.1 Dorsal Root Ganglia

Dorsal root ganglia were isolated from neonatal rats (P4-5) based on a protocol described previously (Mehta et al 2007). Rat pups were anesthetized with isoflourane and sacrificed by decapitation. The ventral skin was incised, and the rib cage and internal organs removed. Starting rostrally, the ventral vertebrae were cut to reveal the entire spinal cord. Dissected DRGs and spinal cords were stored in L-15 media on ice until enzymatic digestion.

Harvested DRGs were incubated in collagenase (2 mg/ml in L15 media) for 30 minutes, followed by 0.25% trypsin (Invitrogen) for 45 minutes, both at 37°C.

Dissociated cells were passed through a 40-µm filter to remove cell aggregates, then incubated on a laminin-coated (10 µg/ml) Petri dish for 1 hour at 37°C. The non-adhered DRGs were collected and diluted in neurobasal media supplemented with 1% glutamax, 0.5% penn strep, 5% FBS, a B27 supplement (Invitrogen), and 100 ng/ml nerve growth factor (NGF, 100 ng/ml; Sigma). Dissociated cells were plated in 48 well plates coated with 10 µg/ml of poly-D-lysine (PDL) at a concentration of 75,000 cells per 250 µl. Ara-C (5 µM) was added for 4 days to minimize astrocyte proliferation.

5.2.1.2 Neonatal OPCs

Spinal cord OPCs were isolated from Sprague Dawley rat pups at P4-5. Dissected spinal cords were minced into small pieces with a scalpel blade (#10) and incubated in collagenase (2 mg/ml) in L15 media for 30-40 minutes at 37°C. The tissue was further digested in 0.25% trypsin with EDTA (Invitrogen) for 20-30 minutes at 37°C and passed through a 40-µm filter. Dissociated OPCs were diluted in proliferation media and plated at 800 cells/ml in flasks coated with PDL (10 µg/ml) until confluence.

For ESTIM experiments, OPCs were co-plated with DRG cultures at 30,00 cells/well (400 cells/mm^2) in differentiation media. One day after plating, co-cultures received one hour of ESTIM at 20 HZ daily for up to 7 days in vitro (DIV).

5.2.1.3 Electrical Stimulation

Lids of 48 well culture plates were retrofitted with pairs of platinum wire electrodes. The electrodes extended into the cell culture media and, to ensure

equal stimulation current in all wells, were connected in series by stainless steel wire. A MultiStim System D-330 stimulator (Digitimer Ltd) (Fields et al 1990; Balkowiec and Katz 2000), connected to the lids with alligator clips, delivered biphasic pulses (6 mA, 200 μs) to induce action potentials in DRGs. Pulses lasted for 500 ms and occurred every 2 sec. Electrodes extended into all wells, but those in control wells were not connected to the stimulator, and therefore did not receive any stimulation.

5.2.1.4 Immunocytochemistry

Co-cultures were fixed with 4% PFA for 45 minutes at 4°C and -20°C methanol for 3 minutes on ice, followed by blocking for 1 hour at room temperature with 10% NGS and 0.4% triton in PBS. Treatment with primary antibodies occurred overnight at 4°C in the blocking solution, followed by incubation with secondary antibodies for 1 hour at room temperature in PBS. Primary antibodies used were: rabbit anti-NG2 (1:1000; Millipore), mouse anti-MBP (1:1000; Calbiochem), and chicken anti-neurofilament (NF, 1:1000; Millipore). Goat secondary antibodies from Jackson Immuno (1:250) included anti-mouse Alexa 488, anti-rabbit Cy3, and anti-chicken Cy5.

5.2.1.5 Cell Quantification

For ESTIM experiments, MBP^+ cells were counted at 20x during a single vertical pass from electrode to electrode in each well (Figure 5.1). Comparison between experimental groups and spinal cord levels was performed using a two- way

ANOVA and Bonferroni post-test (Graphpad Prism). Significance was accepted at $p < 0.05$, and all values are displayed as mean ± SEM.

5.2.2 Animals and FES Treatment

Adult, female Long Evans rats (200-250g upon arrival; Harlan) received a complete spinal cord transection injury at verterbral level T9 as described above. Electrodes were implanted in the tibiailis anterior muscle of both hindlimbs 4 days later, and animals were allowed to recover for 3 days. FES treatment, three 1-hour sessions per day at 20 Hz, began 1 week after injury and lasted either 1 or 7 days (Figure 5.5). Spinal cord tissue from the lumbar enlargement was then harvested for RNA isolation. Experimental groups (n = 3 per group) were:

1) Uninjured control
2) SCI
3) SCI and FES (20 Hz)

5.2.3 Quantitative Real-Time Polymerase Chain Reaction

Co-cultures of DRGs and OPCs received ESTIM for 1, 4, or 7 days in mitogen-free media. Cells from 6 wells were combined and lysed with QIAzol lysis reagent (700 µl; Qiagen 79306). The combined lysates were then homogenized by vortexing for one minute, and the total RNA extracted as described below.

Adult rats were anesthetized as described previously and sacrificed by decapitation. The lumbar enlargement (1.5mm long) was excised and immediately immersed in RNAlater RNA Stabilization Reagent (1 ml; Qiagen 76106) to stabilize RNA. Tissue was stored at -20°C until disruption and homogenization by pestle (Kimble Chase 749515-1500) in QIAzol lysis reagent (700 µl/25 mg tissue; Qiagen 79306).

Total RNA was isolated from tissue and cell homogenates using the miRNeasy Mini kit (Qiagen 217004) and treated with DNase (Qiagen 79254) to remove residual amounts of DNA. The miScript II RT Kit (Qiagen 218161) was used for reverse transcription of total RNA, including microRNA, into cDNA.

MicroRNA and mRNA expression was determined using the miScript SYBR Green PCR Kit (Qiagen 218075). Quantitative real-time polymerase chain reaction was performed according to Qiagen's protocol:

- 15 min PCR initial activation step (95°C)
- 3 step cycling (40 cycles total)
    - 15 sec denaturation (94°C)
    - 30 sec annealing (55°C)
    - 30 sec extension (70°C) – fluorescence data collection
- Melt curve (55°C to 95°C; 0.5°C steps)

The miScript miRNA PCR Array (Qiagen MIRN-107Z) was used to quantify the expression of 86 miRs expressed during development and disease in the rodent nervous system. qRT-PCR using the PCR array was performed on the iCycler

IQ5 (Bio-rad). Individual miScript Primer Assays and QuantiTect Primer Assays (Qiagen) were used to measure expression of OL-specific microRNAs and mRNAs, respectively, isolated from spinal cord tissue. qRT-PCR using individual primers was performed on the CFX Connect™ Real-Time PCR Detection System (Bio-rad 185-5201). Values were normalized to those from control cultures after 1 day in vitro or from naïve, uninjured animals.

Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Measurements were normalized to the expression of U6 small nuclear 2 RNA (RNU6-2) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for miRs and mRNAs, respectively. Comparison between experimental groups was performed using a two-way ANOVA and Bonferroni post-test (Graphpad Prism). Significance was accepted at $p < 0.05$, and all values are displayed as mean ± SEM.

5.3 Results

5.3.1 Mitogen Withdrawal and ESTIM Increase OL Differentiation

Neural activity is required for OPC proliferation in the developing optic nerve (Barres & Raff, 1993), and ESTIM is known to promote OL survival and myelination in vitro (Gary et al., 2012; Malone et al., 2013). However, the role of ESTIM in epigenetic control of OL development is unknown. To determine whether epigenetic mechanisms in OL lineage cells is modulated by neuronal

activity, we cultured P4 rat spinal cord DRGs and OPCs together in mitogen-free media and applied 1 hour of daily ESTIM at 20 Hx for up to one week (Figure 5.1). In control cultures, the number of mature OLs increased from 36.25 ± 1.15 at 1 DIV to 96.75 ± 1.83 at 4 DIV, a 167% change. A further 12% elevation in OL numbers occurred at 7 DIV, bringing the total number of mature OLs to 108 ± 0.54. The same temporal trend was observed in the stimulated cultures, where myelinating OLs numbered 41.25 ± 2.67, 109.25 ± 3.64, and 121 ± 2.46 after 1, 4, and 7 days of ESTIM. Treated wells had 12-13% more MBP^+ OLs than control wells only at 4 and 7 DIV ($p < 0.01$). These findings confirm previous findings (Gary et al., 2012; Malone et al., 2013), indicating that neural activity increases the number of MBP^+ OLs in vitro.

5.3.2 ESTIM Modulates Expression of OL Lineage-Specific microRNAs in DRG/OPC Co-cultures

Next, we analyzed the expression of several components of the miR-17-92 cluster (Figure 5.2), which is known to regulate OPC proliferation (Budde et al., 2010; De Faria Jr et al., 2012). After 4 days in culture, miR-19a and miR-92a were enhanced to 4.21 ± 0.17 (321%) and 4.02 ± 0.32 (302%), respectively, relative to baseline ($p < 0.001$). By the seventh day in culture, the levels of both miRs decreased to 2.59 ± 0.38 and 2.14 ± 0.10, respectively, and were no longer significantly upregulated. Expression of miRs-19b, 20a in control cultures did not change over time. Four doses of ESTIM increased miRs-19a, 20a, and 92a to 3.89 ± 0.47 (289%; $p < 0.001$), 2.99 ± 0.44 (199%; $p < 0.01$), and 2.75 ± 0.38

(175%; $p < 0.01$), respectively, but it suppressed miR-92a expression by 32% versus control ($p < 0.05$). Only miR-92a remained elevated after 7 days of ESTIM at 2.61 ± 0.46 (161%; $p < 0.01$). These findings suggest that the miR-17-92 cluster, previously identified as a promoter of OPC cell proliferation, may also be involved regulating in OL differentiation, and that it can be influenced by neural activity.

Prior studies have identified miRs-138 and miR-338 as modulators of OL differentiation (De Faria Jr et al., 2012; Dugas et al., 2010; Zhao et al., 2010), and miR-23a has been shown to regulate OL myelination (S.-T. Lin & Fu, 2009; S. Lin et al., 2013). We measured the expression of these miRs in DRG/OPC co-cultures treated with daily ESTIM to determine if they play a role in activity-dependent OL development. None of these OL-specific miRs were significantly changed as the OPCs differentiated into mature OLs over 7 days in control cultures (Figure 5.2). Four doses of ESTIM elevated miR-338 levels to 1.97 ± 0.19, a 97% increase relative to the baseline ($p < 0.05$), and a 52% elevation when compared to a single dose (1.36 ± 0.64; $p < 0.01$). miR-338 expression dropped 73% to 0.53 ± 0.35 ($p < 0.001$) after an additional 3 days of ESTIM. Four doses of ESTIM resulted in upregulation of miR-338 expression relative to baseline values ($p < 0.05$) and to a single day of ESTIM ($p < 0.01$). In contrast, miRs-138 and 23a were not influenced by cell maturation or neural activity. This ESTIM-induced upregulation of miR-338 indicates that this miR may play a role in the increased number of MBP^+ OLs observed after 4 stimulation sessions.

5.3.3 Changes in OL-specific miRs are Not Found in Stimulated DRG Cultures

To ensure that the observed changes in miR levels were occurring specifically in OL lineage cells, we stimulated cultures of DRGs alone for 4 days. None of the miRs investigated changed over time or after ESTIM treatment (Figure 5.3). Taken together, these results suggest that alterations in miR levels in the DRG/OPC cultures are restricted to the OL lineage cells. Prior work has shown that neurons are necessary for ESTIM to augment OL numbers in vitro (Gary et al., 2012). Therefore, we did not measure miR levels in cultures of primary OPCs.

5.3.4 microRNA Dsyregulation in the Lumbar Enlargement after Acute SCI

Acute SCI in rodents results in miR dysregulation around the injury site, with the vast majority of miRs suppressed relative to sham animals (Bhalala et al., 2012; N.-K. Liu et al., 2009; Nakanishi et al., 2010; Ryge et al., 2010; Strickland et al., 2011; Yunta et al., 2012; Ziu et al., 2013). To assess if an acute SCI can influence epigenetic mechanisms distant to the injury level, we gave adult female rats a complete transection at T9, and measured miR expression in the lumbar enlargement 8 or 14 days later. As previously reported, the vast majority of miRs studied were downregulated in the lumbar enlargement at one or both time points following the SCI (Figure 5.4). This miR dysregulation in the lumbar

enlargememt indicates that acute SCI can effect epigenetic regulation up to 5 vertebral levels from the injury site.

5.3.5 One Day of FES Treatment Modulates Levels of OL-specific miRs in the Injured Spinal Cord

FES treatment after chronic SCI increases the proliferation of neural precursor cells (Becker et al., 2010) and OPCs (Figure 3.3) in the lumbar spinal cord of adult rats. We sought to determine if microRNAs might mediate these proliferative effects *in vivo*. Adult rats received a complete mid-thoracic transection, followed by 1 or 7 days of FES treatment starting a week after the injury (Figure 5.5). RNA was harvested from the lumbar enlargement, which is expected to have enhanced neural activity as a result of FES.

microRNA-124 is known to regulate NPC proliferation and differentiation (Cheng et al., 2009; Yoo et al., 2009), while miR-9/9* and the miR-17-92 cluster are involved in maintaining OPC numbers (Budde et al., 2010; Lau et al., 2008). SCI did not affect the level of these miRs at either time point (Figure 5.5). One day of FES suppressed miR-124 expression to 0.67 ± 0.06, a 33% decrease relative to baseline ($p < 0.05$), but the inhibition disappeared by the 7th day of treatment. miR-19b, part of the miR-17-92 cluster, was augmented by a single day of FES to 1.16 ± 0.06, a 34% increase versus untreated animals ($p < 0.05$), but returned to control levels after 7 days of stimulation. Neither SCI, nor FES, influenced the expression of miR-9/9* at either time point. Taken together, these results

suggest that a single day of FES treatment may influence OPC proliferation by modulating miR-124 and miR-19b levels.

Several miRs control OL differentiation and myelination, including miRs-138, 219, 338, and 23a (J.-S. Li & Yao, 2012). Eight days after the complete thoracic SCI, miR-338 levels were suppressed to 0.72 ± 0.06 (-28%; $p < 0.05$), and the inhibition continued for up to 2 weeks post-injury (WPI) where miR-338 expression was 0.58 ± 0.07 (-23%; $p < 0.05$) (Figure 5.5). microRNA-23a displayed a similar temporal pattern, dropping to 0.59 ± 0.10 (-41%; $p < 0.05$) and 0.65 ± 0.05 (-35%; $p < 0.05$) at 8 and 14 DPI, respectively. FES treatment delays this inhibition until 2 WPI, when the fold change in expression of miR-338 was 0.72 ± 0.09 (-28%; $p < 0.05$) and miR-23a was 0.59 ± 0.07 (-41%; $p < 0.05$). In contrast, miR-138 and miR-219-1-5p (data not shown) were unchanged after SCI and/or FES treatment. Dicer1, the enzyme that cleaves precursor miR to generate the mature miR transcript, was also unchanged between experimental groups. These observations demonstrate that the FES-mediated return of patterned neural activity to the lumbar spinal cord can influence miR-338 and miR-23a levels, possibly influencing the extent of OL differentiation and remyelination after SCI.

5.3.6 Neural Activity Upregulates DNMT Expression After Acute SCI

Additional ways of regulating OL development epigenetically include DNA methylation and chromatin remodeling. Developing nervous system tissue has

elevated levels of DNA methyltransferases (DNMTs), which decline during differentiation (Simmons et al., 2013), and synaptic activity increases the extent of DNA methylation (Levenson et al., 2006). Histone deacetylases (HDACs) alleviate negative regulation of OPC differentiation by removing acetyl groups from chromatin, thereby promoting OL lineage progression (Conway, O'Bara, Vedia, Pol, & Sim, 2012; Fancy et al., 2011; Wood et al., 2013; Ye et al., 2009). We used qRT-PCR to assess mRNA expression of the major DNMTs (DNMT1, 3a, and 3b) and HDAC1/2 in the lumbar spinal cord of adult rats after a complete acute transection at T9 and FES treatment (Figure 5.6). Of the 3 DNMTs investigated, only DNMT1 was influenced by SCI, rising 37% to 1.37 ± 0.04 at 2 WPI ($p < 0.01$). One day of FES increased DNMT1 expression to 1.29 ± 0.09, a 29% change from baseline ($p < 0.05$) and a 26% growth relative to control animals ($p < 0.05$). Short-term stimulation had a similar effect on DNMT3b levels, which rose to 1.47 ± 0.21, representing a 47% and 57% enhancement versus baseline ($p < 0.01$) and unstimulated ($p < 0.05$) animals, respectively. Expression of HDACs1 and 2 was not influenced by SCI or FES treatment. These observations indicate that FES can augment DNMT activity after an acute SCI.

Figure 5.1. ESTIM promotes myelination in DRG/OPC co-cultures.

(A) Schematic of the experimental time line. (B – D) Representative images of of co-cultures at (B) 1, (C) 4, and (D) 7 DIV. Scale bars = 25 μm. (E) Schematic of cell quantification during a single vertical scan from electrode to electrode. (F) Quantification of MBP^+ cells. Data presented as mean ± SEM. **$p < 0.01$ (two-way ANOVA with Bonferoni post-test)

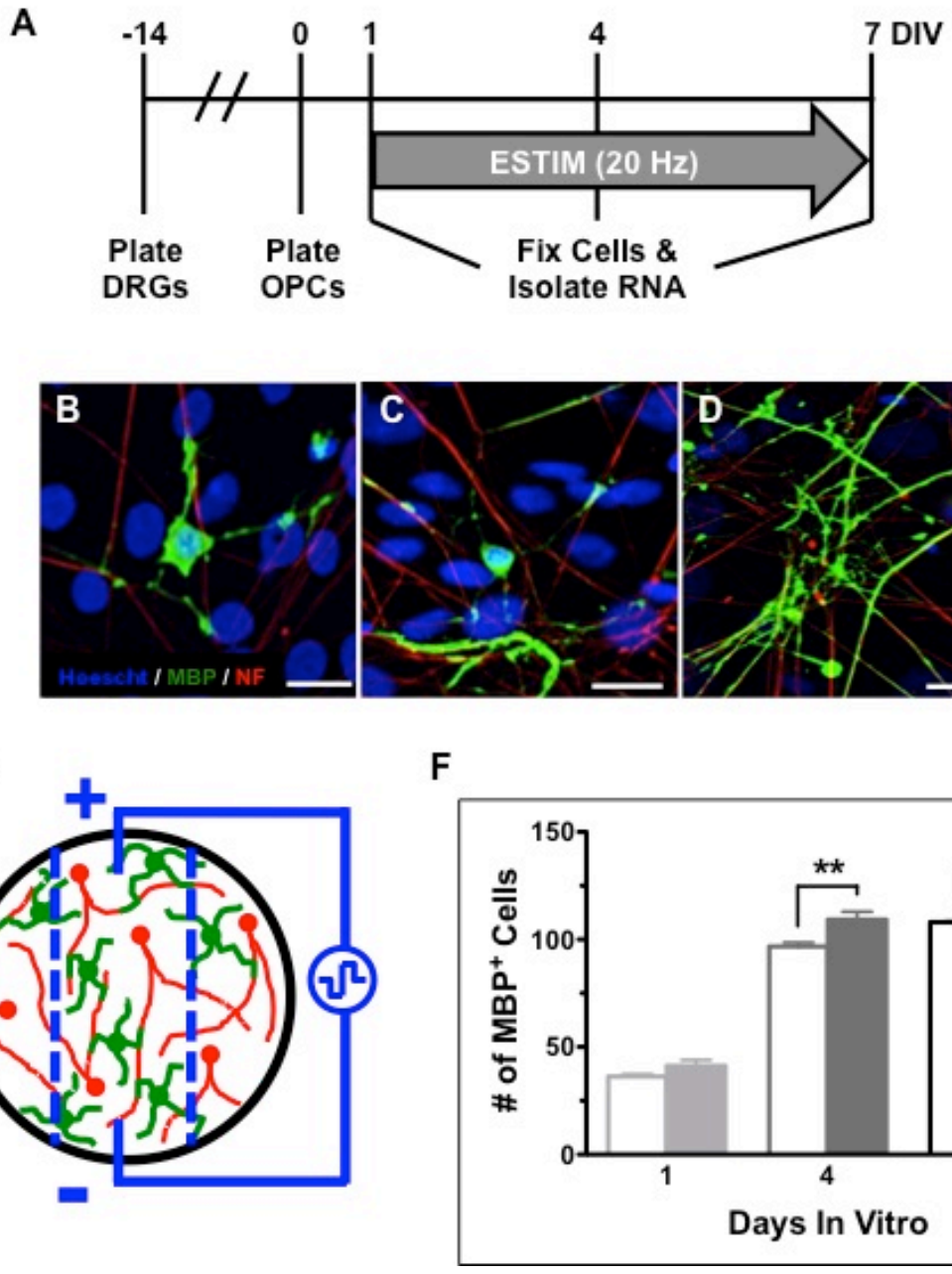

A
-14
0
1
4
7 DIV
ESTIM (20 Hz)
Plate DRGs
Plate OPCs
Fix Cells & Isolate RNA
B
C
D
Hoescht / MBP / NF
E
+
-
F
150
100
50
0
# of MBP+ Cells
**
**
1
4
7
Days In Vitro

Figure 5.2. ESTIM alters expression of OL-specific miRs.

Quantification of (A) the miR-17-92 cluster and (B) miRs-138, 338, and 23a in OPC/DRG co-cultures treated with daily ESTIM at 20 Hz for up to 1 week. Expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to control values at 1 DIV. RNU6-2 was used as an internal control. Data represent mean ± SEM. P-values determined using two-way ANOVA followed by the Bonferoni post-test. *p < 0.05, **p < 0.01, ***p < 0.001.

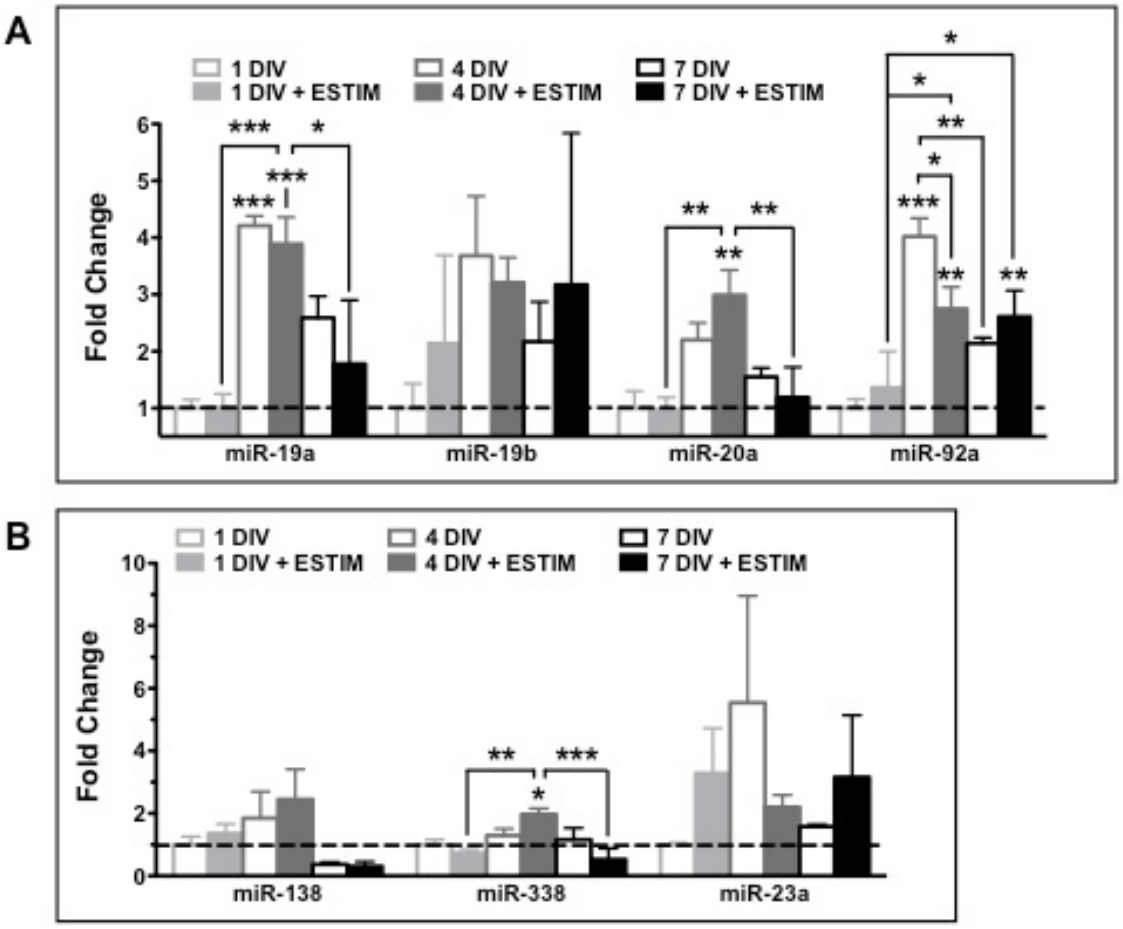


Figure 5.3. ESTIM does not influence miR expression in primary DRG cultures.

Quantification of miR expression in cultures of primary DRGs treated with daily ESTIM at 20 Hz for up to 4 days. Neural activity does not influence the expression of (A) the miR-17-92 cluster or (F) miRs-138, miR-338, or miR-23a. Expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to control values at 1 DIV. RNU6-2 was used as an internal control. Data represent mean ± SEM. P-values determined using two-way ANOVA followed by the Bonferoni post-test (n = 3).

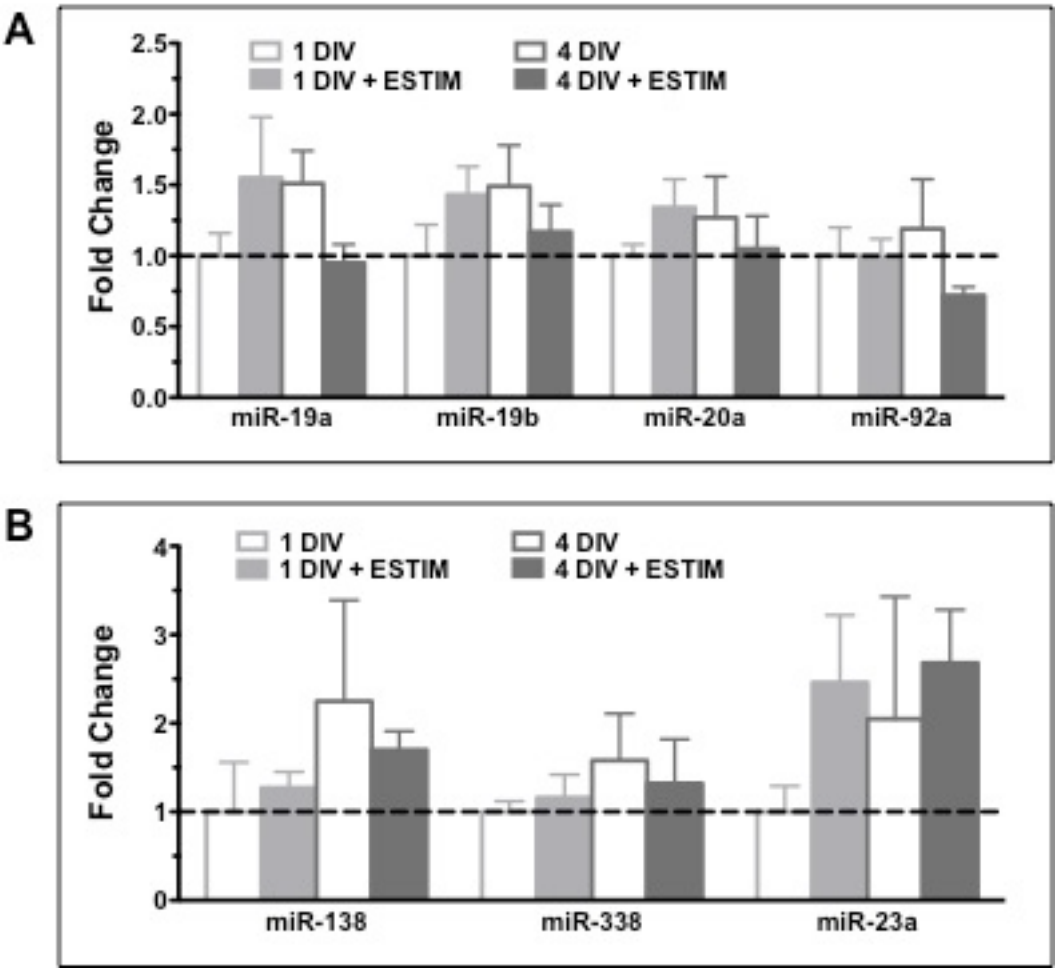

A
1 DIV
1 DIV + ESTIM
4 DIV
4 DIV + ESTIM
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
miR-19a
miR-19b
miR-20a
miR-92a
B
1 DIV
1 DIV + ESTIM
4 DIV
4 DIV + ESTIM
Fold Change
4
3
2
1
0
miR-138
miR-338
miR-23a

Figure 5.4. miR dysregulation in the lumbar enlargement after acute SCI. microRNAs that are (A) upregulated or (B – C) suppressed following acute SCI. Expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to uninjured control animals. RNU6-2 was used as an internal control. Data represent mean ± SEM.

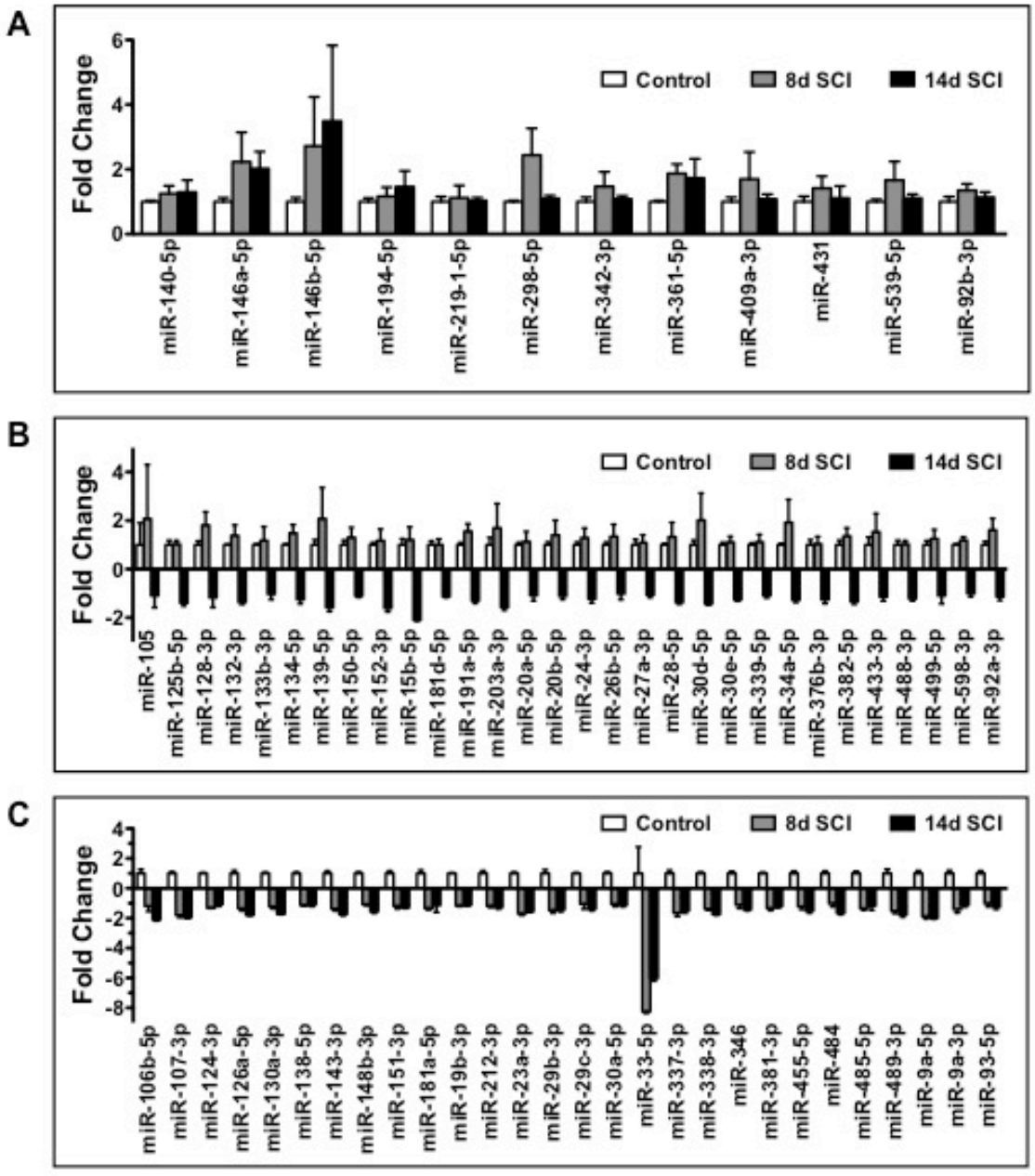


Figure 5.5. FES alters expression of OL-specific miRs.

(A) Schematic of the experimental timeline. Quantification of OL-specific miRs that regulate (B) OPC proliferation and (C) OL maturation. (D) Levels of Dicer1 mRNA after SCI and FES treatment. Expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to uninjured control animals. RNU6-2 and GAPDH were used as internal controls for miRs and mRNA, respectively. Data represent mean ± SEM. *$p < 0.05$ (two-way ANOVA with Bonferoni post-test)

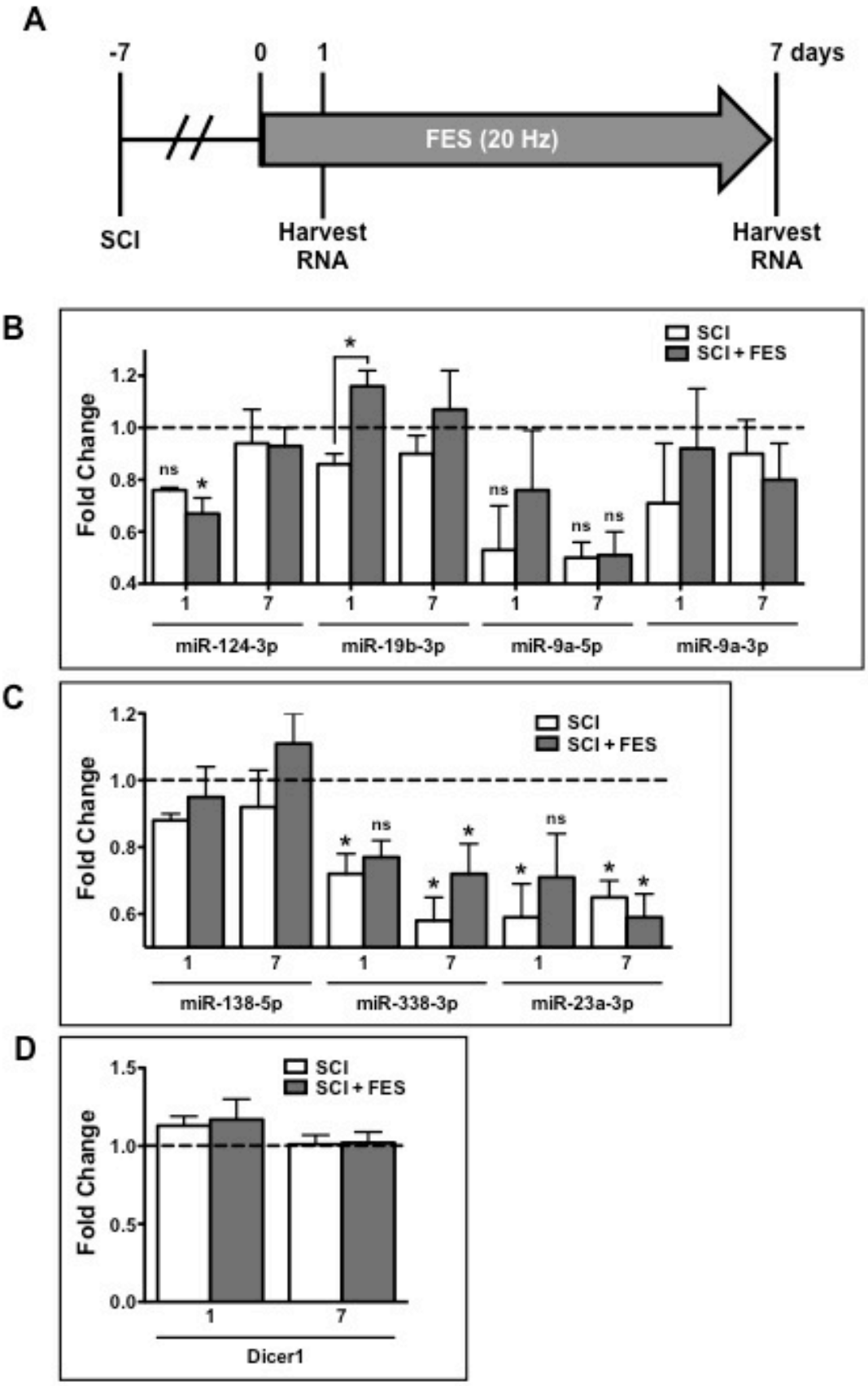
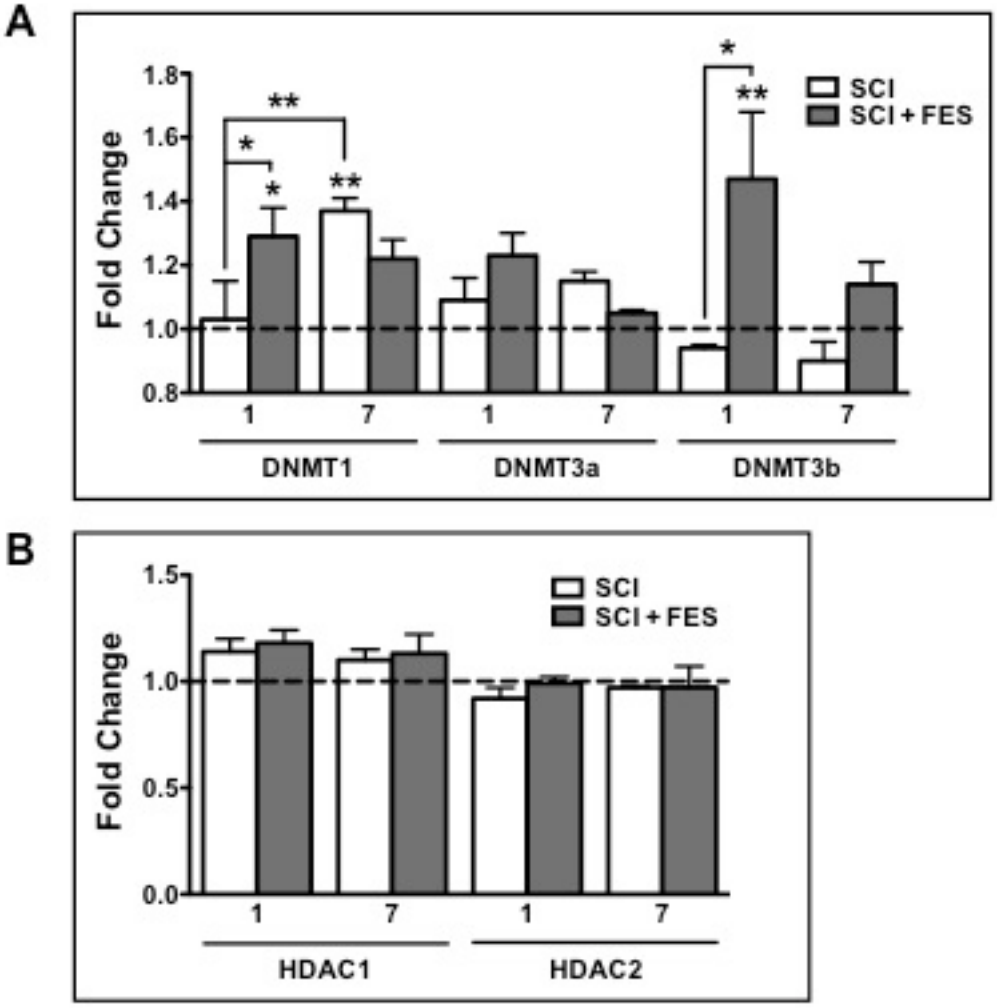

A
-7
0
1
7 days
FES (20 Hz)
SCI
Harvest RNA
Harvest RNA
B
SCI
SCI + FES
Fold Change
1.2
1.0
0.8
0.6
0.4
ns
*
ns
*
ns
ns
1
7
1
7
1
7
1
7
miR-124-3p
miR-19b-3p
miR-9a-5p
miR-9a-3p
C
SCI
SCI + FES
Fold Change
1.2
1.0
0.8
0.6
*
ns
*
*
*
ns
*
1
7
1
7
1
7
miR-138-5p
miR-338-3p
miR-23a-3p
D
SCI
SCI + FES
Fold Change
1.5
1.0
0.5
0.0
1
7
Dicer1

Figure 5.6. Expression of DNMT1/3b is upregulated by FES treatment. mRNA expression levels of (A) DNA methyltransferases and (B) histone deacetylases in the lumbar enlargement after 1 or 7 days of FES treatment at 20 Hz. Expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to uninjured control animals. GAPDH was used as the internal controls. P-values determined using two-way ANOVA followed by the Bonferoni post-test (n = 3). *p < 0.05, **p < 0.01.


A
Fold Change
1.8
1.6
1.4
1.2
1.0
0.8
SCI
SCI + FES
*
**
1
7
DNMT1
DNMT3a
DNMT3b
B
1.5
1.0
0.5
0.0
HDAC1
HDAC2

Chapter 6: Discussion

6.1 Frequency-dependent Regulation of OL Development in the Chronically Injured Adult Spinal Cord

The frequency-dependent effects of ESTIM on cells of the nervous system have been demonstrated previously (Balkowiec & Katz, 2000; Gary et al., 2012; B Stevens et al., 1998). In co-cultures of Schwann cells and DRGs, myelination was suppressed by ESTIM at 0.1 Hz, but unaffected by 1 Hz stimulation (B Stevens et al., 1998). Electrical stimulation at 10 Hz, but not 1 or 100 Hz, increased the number of MBP^+ OLs in cultures of rat cortical neurons and OPCs (Gary et al., 2012). We found that the number of $BrdU^+$ cells in the injured spinal cord of adult rats is elevated by FES at 20 Hz or 100 Hz, while the number of $BrdU^+/NG2^+$ cells, indicative of OPC proliferation, is significantly higher after FES at 20 Hz group. These findings indicate that 20 Hz is the optimal stimulation frequency to maximize the number of proliferating OPCs after SCI.

Neural activity promotes OPC differentiation (Q. Li et al., 2010), in addition to OL myelination and viability (Gary et al., 2012; Malone et al., 2013), through several mechanisms, including purinergic signaling (Beth Stevens, Porta, Haak, Gallo, & Fields, 2002) and activity-induced secretion of LIF from astrocytes (Ishibashi et

al., 2006). We determined that the number of $BrdU^+$ OLs in the chronically transected rat spinal cord was not significantly changed after FES treatment, regardless of stimulation frequency. In contrast, Li et al. (2010) found increased BrdU incorporation in dorsal column OLs after ESTIM of the contralateral pyramidal. The lack of an FES-induced effect on OPC differentiation in our experiments may be due to the animal survival time after BrdU injection. OPC division rates in the rodent cortex, corpus callosum, optic nerve, and spinal cord decline with age (Young et al., 2013). By P60, the total cell cycle time for OPCs in spinal cord white matter increases to ~15 days (Young et al., 2013), likely due to an extended G1 phase (Simon, Götz, & Dimou, 2011). Although CNS injury causes adult OPCs to proliferate 5x faster than normal (Simon et al., 2011), it also delays their differentiation into mature OLs (Sim, Zhao, Penderis, & Franklin, 2002). Taken together, these findings suggest that additional survival time after BrdU pulse labeling might be necessary to observe increased OPC differentiation after FES.

6.2 Proliferation and Differentiation of Harvested OPCs after Short-term FES

The effect of ESTIM on glial cells is dependent on the stimulation frequency and the number of stimulation sessions. Although 1 hour of ESTIM is sufficient to increase both the onset and extent of Schwann cell myelination of DRG axons *in vitro* and *in vivo* (Wan et al., 2010), at least 3 daily 1-hour sessions are required

to promote myelination in OPC/DRG co-cultures (Malone et al., 2013). We found that 3 FES sessions (20 Hz), delivered on the 7th day after an acute thoracic transection, increased the differentiation of OPCs harvested from the lumbar enlargement and cultured in mitogen-free media. However, previous work shows ESTIM promotes OL viability and myelination without influencing differentiation (Gary et al., 2012; Malone et al., 2013). These conflicting results may be due to differences in experimental design. Gary et al. used embryonic mixed cortical cultures, and let the primary OPCs mature for 3 days prior to ESTIM application, which is enough time for the vast majority of OPCs to differentiate into $GalC^{+}MBP^{+}$ OLs (Dugas, Tai, Speed, Ngai, & Barres, 2006).

Our experiments also show that short-term FES does not increase proliferation of harvested adult OPCs in the presence of PDGF, suggesting that additional days of stimulation might be necessary to enhance the proliferative ability of these cells. However, both PDGF and bFGF are required to keep adult OPCs from the spinal cord or optic nerve require to remain actively dividing and undifferentiated *in vitro* (Engel & Wolswjk, 1996; Wolswjk & Noble, 1992). In the presence of PDGF alone, adult OPCs divide slowly and give rise to OLs by asymmetric division and differentiation (Engel & Wolswjk, 1996). This suggests that the lack of a difference in OPC proliferation between the control and stimulated animals is due to the absence of a key mitogen in the proliferation media. Further work is needed to determine if short-term FES can influence the division rate of adult-born OPCs.

6.3 Activity-dependent Regulation of Epigenetic Mechanisms in Oligodendroglial Cells

The degree of neurological impairment after SCI depends on the extent of the secondary damage after the initial injury, functionality of remaining tissue, and endogenous repair mechanisms such as remyelination. Studying the molecular changes that occur in OL lineage cells is paramount in understanding the role of activity-based therapy after SCI. Neural activity has been implicated in regulating OL biology and miR expression (Eacker, Dawson, & Dawson, 2013; Eacker, Keuss, Berezikov, Dawson, & Dawson, 2011; Nudelman et al., 2010; Ryan, Donovan, & McLoughlin, 2013; Wibrand et al., 2010). Our experiments are the first to show that neural activity modulates OL-specific miRs in OPC/DRG co-cultures and in the injured adult rodent spinal cord.

6.3.1 Modulation of Epigenetic Mechanisms *In Vitro*

6.3.1.1 The miR-17-92 Cluster

Mitogen withdrawal is known to induce OPC differentiation *in vitro* by upregulating miRs-219, -138, and -338 (Dugas et al., 2010). Our experiments are the first to demonstrate that OPC differentiation after mitogen withdrawal also elevates the expression of miR-17-92 cluster components. In contrast, De Faria et al. (2012) showed that levels of miR-20a and miR-92a are reduced at 5 and 8 DIV, while miR-19a and miR-19b aren't suppressed until 8 DIV, in primary cultures of cortical OPCs from P0 rats. Differences between our data and these

published results might be due to the anatomical origin of the cells and the media components. Dicer1 ablation, under the control of the CNP promoter, has different effects on the number of $Olig2^+$ cells in the P0 rodent brain and spinal cord, suggesting that OL development is regulated by distinct mechanisms in these anatomical regions (Budde et al., 2010). Additionally, the media used in De Faria's study contained triiodothyronine (T3), which exacerbates the effect of mitogen-withdrawal on miR expression in OL lineage cells (Dugas et al., 2010).

These data are the first to show that ESTIM influences expression of OL-specific miRs in co-cultures of OPCs and DRG, and that the changes in expression were dependent on the number of ESTIM sessions. Four daily sessions of ESTIM were required to upregulate components of the miR-17-92 cluster, supporting the conclusion that ESTIM-mediated effects in OL lineage cells are a function of session number (Malone et al., 2013). In addition to promoting cell division, increased expression of miR-20a and miR-92a also enhances cell viability by preventing apoptosis in cancer cells (Niu et al., 2012; Sharifi, Salehi, Gheisari, & Kazemi, 2013, 2014; Tian, Fang, Xue, & Chen, 2013; Q. Wu et al., 2013). The elevation of these miRs after ESTIM treatment may have a similar effect on the OL lineage cells in our experiments, possibly underlying the increased number of mature OLs observed in stimulated cultures from this study and from previous work (Gary et al., 2012).

We also found that the miR-17-92 cluster is regulated temporally by both mitogen withdrawal and ESTIM treatment. microRNA-20a modulates expression of the E2F family of transcription factors (Sylvestre et al., 2007), which controls OPC

cell cycle exit (Magri et al., 2014). E2F1-3 binds to the miR-17-92 promoter, creating a feedback loop to prevent precocious cell proliferation (Sylvestre et al., 2007). These findings suggest that a similar feedback loop is present in oligodendrolia and is responsible for the temporal regulation of the miR-17-92 *in vitro*.

6.3.1.2 Differentiation- and Myelination-Promoting miRs

Increased expression of miR-138 and miR-338 initiates the transition from cycling OPC to immature OL by targeting PDGRFa, FGFR2, Hes5, Sox6, and UHRF1bp1, while elevated miR-23a promotes myelination by inhibiting laminB1 (Dugas & Notterpek, 2011). We found that these miRs don't change significantly as OPCs mature over time, in sharp contrast to prior studies (De Faria Jr et al., 2012; Dugas et al., 2010). In addition, ESTIM only upregulated miR-338 after 4 days of treatment, while miR-138 and miR-23a were unaffected at all time points, despite the increased number of MBP+ OLs observed after 4 and 7 days of ESTIM. These unexpected results might be due to miR expression in the DRG cell bodies and/or axons. The miRs mentioned above are considered to be specific to the OL lineage because their expression is much higher in OLs than in astrocytes, neurons, or DRGs (Budde et al., 2010; Dugas et al., 2010; Hua et al., 2009). However, our data show that they are still detectable in primary cultures of DRGs. We attempted to rule out effects of miR changes in DRGs by quantifying miR expression in primary DRG cultures with and without ESTIM, and found that none of the miRs mentioned above changed significantly over time or in response to stimulation. However, OL lineage cells can communicate with

axons via neurotransmitter-mediated release of exosomes (Frühbeis et al., 2013). These exosomes contain proteins and RNA, includning miRs (Pusic & Kraig, 2014), possibly influencing miR expression in the DRGs that aren't evident when DRGs are stimulated alone. Therefore it is possible that our results may be due to variations in miR levels in DRGs. Further work is required to identify activity-dependent miR changes specifically in OL lineage cells treated with ESTIM.

6.3.2 Modulation of Epigenetic Mechanisms *In Vivo*

Following an acute SCI in adult rats, the miR network is dysregulated around the lesion site (N.-K. Liu et al., 2009; Nakanishi et al., 2010; Strickland et al., 2011; Yunta et al., 2012; Ziu et al., 2013). Our experiments show that this dysregulation also occurs 4-8 levels caudal to the lesion for up to 14 DPI. The majority of miRs in our experiments were downregulated, in accordance with previous studies (N.-K. Liu et al., 2009; Strickland et al., 2011; Yunta et al., 2012). However, our data are the first to show that alterations in miR levels after acute SCI are independent of Dicer1, the protein that synthesizes mature miRs, expression.

6.3.2.1 Lineage Specification and Cell Proliferation

In the first few weeks after SCI, endogenous OPCs migrate to the lesion site and proliferate extensively and replace OLs lost to necrosis and apoptosis. Previous studies have observed upregulation of miR-17-92 cluster components miR-17, miR-20, and miR-19a within and around the lesion (N.-K. Liu et al., 2009; Yunta

et al., 2012), which may contribute to the increased OPC proliferation typically observed after SCI. Our data show that these alterations do not extend caudally into the lumbar enlargement within the first 2 weeks after injury. This suggests that the dysregulation of the miR-17-92 cluster may play a role in the spatial regulation of OPC proliferation after actue SCI. We also found that one day of FES treatment increases the expression of miR-19b. Prior work has shown that miR-19b, the most abundant component of the miR-17-92 cluster, enhances OPC proliferation through Pten/Akt signaling (Budde et al., 2010). Taken together, these findings indicate that the FES-induced elevation in miR-19b may promote OPC proliferation in the injured spinal cord, and might be responsible for the increased OPC division we observed after FES treatment in chronically injured animals.

6.3.2.2 Differentiation- and Myelination-Promoting miRs

Numerous factors contribute to OL death from necrosis and apoptosis after SCI, including ischemia, inflammation, glutatamate excitotoxicity, hypoxia, and free radicals (Mekhail, Almazan, & Tabrizian, 2012). In animal models of SCI, the resulting demyelination is observed within the first 24 hours after injury, followed by a second wave of OL death and enhanced demyelination 3 weeks PI (Griffiths & McCulloch, 1983; Totoiu & Keirstead, 2005). By 14 DPI, OL apoptosis extends several millimeters rostrocaudally from the injury site, while neuronal apoptosis is restricted to the lesion (Crowe, Bresnahan, Shuman, Masters, & Beattie, 1997; X. Z. Liu et al., 1997). These findings suggest that the decreased expression of miR-338 and miR-23a that we observed at 8 and 14 DPI is due to elevated OL

death and demyelination after SCI. However, miR-138 and miR-219 are not influenced by SCI, contradicting this theory. ESTIM promotes OL viability and survival in vitro, resulting in increased numbers of MBP^+ OLs in mixed cortical cultures (Gary et al., 2012). FES may have a similar effect in vivo, possibly delaying miR-338 and miR-23a inhibition by enhancing OL viability in the injured spinal cord.

6.3.2.3 DNA Methylation

As discussed previously, DNMTs and HDACs can also influence OL development. Our data show that SCI induces DNMT1 activity, but not until 14 DPI. In contrast, FES treatment promotes both earlier and elevated DNMT1 expression at 7 DPI. Since DNMT1 is a maintenance DNMT, responsible for passing on established methylation patterns to the newly replicated DNA in daughter cells (MacDonald & Roskams, 2009), its FES-induced increase is possibly due to enhanced division of OPCs. DNMT1 activity in NPCs is also required for neurogenesis at the expense of astrocyte differentiation (Juliandi et al., 2010), suggesting that it might be responsible for the increased number of $nestin^+$ cells found after FES treatment (Becker et al., 2010). However, DNMT1 suppression is neuroprotective after ischemia-induced injury (MacDonald & Roskams, 2009), underscoring the complicated role it plays after CNS injury.

6.4 Concluding Remarks

Currently, most research initiatives for improving remyelintion focus on the transplantation of myelinating cells into the injury site or delivering factors that increase OL survival and development. However, FES provides a noninvasive option for generating neural activity in the injured nervous system to regulate OL biology. Functional remyelination following CNS injury or disease may require manipulation of all the phases of OL development in order to replace OLs and successfully ensheath spared axons. Therefore it is important to assess the intrinsic molecular changes that occur in OL lineage cells in the injured CNS, and how they are influenced by neural activity.

Bibliography

Ahn, Y. H., Lee, G., & Kang, S. K. (2006). Molecular insights of the injured lesions of rat spinal cords: Inflammation, apoptosis, and cell survival. *Biochemical and Biophysical Research Communications*, *348*(2), 560–70. doi:10.1016/j.bbrc.2006.07.105

Back, S. A., Luo, N. L., Borenstein, N. S., Volpe, J. J., & Kinney, H. C. (2002). Arrested oligodendrocyte lineage progression during human cerebral white matter development: dissociation between the timing of progenitor differentiation and myelinogenesis. *Journal of Neuropathology and Experimental Neurology*, *61*(2), 197–211. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11853021

Balasubramaniyan, V., Boddeke, E., Bakels, R., Küst, B., Kooistra, S., Veneman, a, & Copray, S. (2006). Effects of histone deacetylation inhibition on neuronal differentiation of embryonic mouse neural stem cells. *Neuroscience*, *143*(4), 939–51. doi:10.1016/j.neuroscience.2006.08.082

Balkowiec, a, & Katz, D. M. (2000). Activity-dependent release of endogenous brain-derived neurotrophic factor from primary sensory neurons detected by ELISA in situ. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *20*(19), 7417–23. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11007900

Baltan, S., Bachleda, A., Morrison, R. S., & Murphy, S. P. (2011). Expression of Histone Deacetylases in Cellular Compartments of the Mouse Brain and the Effects of Ischemia. *Translational Stroke Research*, *2*(3), 411–423. doi:10.1007/s12975-011-0087-z.Expression

Baltan, S., Murphy, S. P., Danilov, C. a, Bachleda, A., & Morrison, R. S. (2011). Histone deacetylase inhibitors preserve white matter structure and function during ischemia by conserving ATP and reducing excitotoxicity. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *31*(11), 3990–9. doi:10.1523/JNEUROSCI.5379-10.2011

Bansal, R., & Pfeiffer, S. E. (1997). FGF-2 converts mature oligodendrocytes to a novel phenotype. *Journal of Neuroscience Research*, *50*(2), 215–28. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9373031

Barca-mayo, O., & Lu, Q. R. (2012). Fine-tuning oligodendrocyte development by microRNAs. *Neuroscience Research*, *6*(February), 1–7. doi:10.3389/fnins.2012.00013

Barnabé-Heider, F., Göritz, C., Sabelström, H., Takebayashi, H., Pfrieger, F. W., Meletis, K., & Frisén, J. (2010). Origin of new glial cells in intact and injured adult spinal cord. *Cell Stem Cell*, *7*(4), 470–82. doi:10.1016/j.stem.2010.07.014

Baron, W., Shattil, S. J., & ffrench-Constant, C. (2002). The oligodendrocyte precursor mitogen PDGF stimulates proliferation by activation of alpha(v)beta3 integrins. *The EMBO Journal*, *21*(8), 1957–66. doi:10.1093/emboj/21.8.1957

Barres, B., & Raff, M. (1993). Proliferation of oligodendrocyte precursor cells depends on electrical activity in axons. *Nature*, *361*, 258–260. Retrieved from http://www.nature.com/nature/journal/v361/n6409/abs/361258a0.html

Bashtrykov, P., Jankevicius, G., Jurkowska, R. Z., Ragozin, S., & Jeltsch, A. (2013). The Uhrf1 protein stimulates the activity and specificity of the maintenance DNA methyltransferase Dnmt1 by an allosteric mechanism. *The Journal of Biological Chemistry*, 1–18. doi:10.1074/jbc.M113.528893

Beaumont, E., Guevara, E., Dubeau, S., Lesage, F., Nagai, M., & Popovic, M. (2013). Functional electrical stimulation post-spinal cord injury improves locomotion and increases afferent input into the central nervous system in rats. *The Journal of Spinal Cord Medicine*, *0*(0), 1–8. doi:10.1179/2045772313Y.0000000117

Becker, D., Gary, D. S., Rosenzweig, E. S., Grill, W. M., & McDonald, J. W. (2010). Functional electrical stimulation helps replenish progenitor cells in the injured spinal cord of adult rats. *Experimental Neurology*, *222*(2), 211–8. doi:10.1016/j.expneurol.2009.12.029

Belegu, V., Oudega, M., Gary, D. S., & McDonald, J. W. (2007). Restoring function after spinal cord injury: promoting spontaneous regeneration with stem cells and activity-based therapies. *Neurosurgery Clinics of North America*, *18*(1), 143–68, xi. doi:10.1016/j.nec.2006.10.012

Bergles, D. E., Jabs, R., & Steinhäuser, C. (2010). Neuron-glia synapses in the brain. *Brain Research Reviews*, *63*(1-2), 130–7. doi:10.1016/j.brainresrev.2009.12.003

Bhalala, O. G., Pan, L., Sahni, V., McGuire, T. L., Gruner, K., Tourtellotte, W. G., & Kessler, J. a. (2012). microRNA-21 regulates astrocytic response following spinal cord injury. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *32*(50), 17935–47. doi:10.1523/JNEUROSCI.3860-12.2012

Bhalala, O. G., Srikanth, M., & Kessler, J. a. (2013). The emerging roles of microRNAs in CNS injuries. *Nature Reviews. Neurology*, *9*(6), 328–39. doi:10.1038/nrneurol.2013.67

Bradbury, E. J., & Carter, L. M. (2011). Manipulating the glial scar: chondroitinase ABC as a therapy for spinal cord injury. *Brain Research Bulletin*, *84*(4-5), 306–16. doi:10.1016/j.brainresbull.2010.06.015

Bramlett, H. M., & Dietrich, W. D. (2007). Progressive damage after brain and spinal cord injury: pathomechanisms and treatment strategies. *Progress in Brain Research*, *161*, 125–41. doi:10.1016/S0079-6123(06)61009-1

Budde, H., Schmitt, S., Fitzner, D., Opitz, L., Salinas-Riester, G., & Simons, M. (2010). Control of oligodendroglial cell number by the miR-17-92 cluster. *Development (Cambridge, England)*, *137*(13), 2127–32. doi:10.1242/dev.050633

Buller, B., Chopp, M., Ueno, Y., Zhang, L., Zhang, R. L., Morris, D., … Zhang, Z. G. (2012). Regulation of serum response factor by miRNA-200 and miRNA-9 modulates oligodendrocyte progenitor cell differentiation. *Glia*, *60*(12), 1906–14. doi:10.1002/glia.22406

Cai, J., Qi, Y., Hu, X., Tan, M., Liu, Z., Zhang, J., … Qiu, M. (2005). Generation of oligodendrocyte precursor cells from mouse dorsal spinal cord independent of Nkx6 regulation and Shh signaling. *Neuron*, *45*(1), 41–53. doi:10.1016/j.neuron.2004.12.028

Cheng, L.-C., Pastrana, E., Tavazoie, M., & Doetsch, F. (2009). miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. *Nature Neuroscience*, *12*(4), 399–408. doi:10.1038/nn.2294

Cohen, J. E., & Fields, R. D. (2008). Activity-dependent neuron-glial signaling by ATP and leukemia-inhibitory factor promotes hippocampal glial cell development. *Neuron Glia Biology*, *4*(1), 43–55. doi:10.1017/S1740925X09000076

Conway, G. D., O'Bara, M. a, Vedia, B. H., Pol, S. U., & Sim, F. J. (2012). Histone deacetylase activity is required for human oligodendrocyte progenitor differentiation. *Glia*, *60*(12), 1944–53. doi:10.1002/glia.22410

Crowe, M. J., Bresnahan, J. C., Shuman, S. L., Masters, J. N., & Beattie, M. S. (1997). Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. *Nature Medicine*, *3*(1), 73–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8986744

Czopka, T., Ffrench-Constant, C., & Lyons, D. A. (2013). Individual oligodendrocytes have only a few hours in which to generate new myelin sheaths in vivo. *Developmental Cell*, *25*(6), 599–609. doi:10.1016/j.devcel.2013.05.013

Dash, P. K., Orsi, S. a, Zhang, M., Grill, R. J., Pati, S., Zhao, J., & Moore, A. N. (2010). Valproate administered after traumatic brain injury provides neuroprotection and improves cognitive function in rats. *PloS One*, *5*(6), e11383. doi:10.1371/journal.pone.0011383

Davis, G. M., Hamzaid, N. a., & Fornusek, C. (2008). Cardiorespiratory, metabolic, and biomechanical responses during functional electrical stimulation leg exercise: health and fitness benefits. *Artificial Organs*, *32*(8), 625–629. doi:10.1111/j.1525-1594.2008.00622.x

De Biase, L. M., Kang, S. H., Baxi, E. G., Fukaya, M., Pucak, M. L., Mishina, M., … Bergles, D. E. (2011). NMDA Receptor Signaling in Oligodendrocyte Progenitors Is Not Required for Oligodendrogenesis and Myelination. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *31*(35), 12650–62. doi:10.1523/JNEUROSCI.2455-11.2011

De Biase, L. M., Nishiyama, A., & Bergles, D. E. (2010). Excitability and synaptic communication within the oligodendrocyte lineage. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *30*(10), 3600–11. doi:10.1523/JNEUROSCI.6000-09.2010

De Castro, F., & Bribián, A. (2005). The molecular orchestra of the migration of oligodendrocyte precursors during development. *Brain Research. Brain Research Reviews*, *49*(2), 227–41. doi:10.1016/j.brainresrev.2004.12.034

De Faria Jr, O., Cui, Q., Bin, J. M., Bull, S., Kennedy, T. E., Bar-or, A., & P, J. (2012). Regulation of miRNA 219 and miRNA clusters 338 and 17-92 in oligodendrocytes. *Biosystems*, *3*(March), 1–13. doi:10.3389/fgene.2012.00046

Demerens, C., Stankoff, B., Logak, M., Anglade, P., Allinquant, B., Couraud, F., … Lubetzki, C. (1996). Induction of myelination in the central nervous system by electrical activity. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(18), 9887–92. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=38524&tool=pmcentrez&rendertype=abstract

Devivo, M. J. (2012). Epidemiology of traumatic spinal cord injury: trends and future implications. *Spinal Cord*, *50*(5), 365–72. doi:10.1038/sc.2011.178

Dietz, V. (2010). Behavior of spinal neurons deprived of supraspinal input. *Nature Reviews. Neurology*, *6*(3), 167–74. doi:10.1038/nrneurol.2009.227

Dietz, V. (2012). Neuronal plasticity after a human spinal cord injury: positive and negative effects. *Experimental Neurology*, *235*(1), 110–5. doi:10.1016/j.expneurol.2011.04.007

Dugas, J. C., Cuellar, T. L., Scholze, A., Ason, B., Ibrahim, A., Emery, B., … Barres, B. A. (2010). Dicer1 and miR-219 Are Required for Normal Oligodendrocyte Differentiation and Myelination. *Neuron*, *65*(5), 597–611. doi:10.1016/j.neuron.2010.01.027

Dugas, J. C., & Notterpek, L. (2011). MicroRNAs in oligodendrocyte and Schwann cell differentiation. *Developmental Neuroscience*, *33*(1), 14–20. doi:10.1159/000323919

Dugas, J. C., Tai, Y. C., Speed, T. P., Ngai, J., & Barres, B. a. (2006). Functional genomic analysis of oligodendrocyte differentiation. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *26*(43), 10967–83. doi:10.1523/JNEUROSCI.2572-06.2006

Dumont, R. J., Okonkwo, D. O., Verma, S., Hurlbert, R. J., Boulos, P. T., Ellegala, D. B., & Dumont, a S. (2001). Acute spinal cord injury, part I: pathophysiologic mechanisms. *Clinical Neuropharmacology*, *24*(5), 254–64. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11586110

Duncan, I. D., Brower, a, Kondo, Y., Curlee, J. F., & Schultz, R. D. (2009). Extensive remyelination of the CNS leads to functional recovery. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(16), 6832–6. doi:10.1073/pnas.0812500106

Eacker, S. M., Dawson, T. M., & Dawson, V. L. (2013). The interplay of microRNA and neuronal activity in health and disease. *Frontiers in Cellular Neuroscience*, *7*(August), 136. doi:10.3389/fncel.2013.00136

Eacker, S. M., Keuss, M. J., Berezikov, E., Dawson, V. L., & Dawson, T. M. (2011). Neuronal activity regulates hippocampal miRNA expression. *PloS One*, *6*(10), e25068. doi:10.1371/journal.pone.0025068

Edgerton, V. R., Leon, R. D., Harkema, S. J., Hodgson, J. a, London, N., Reinkensmeyer, D. J., … Tobin, a. (2001). Retraining the injured spinal cord. *The Journal of Physiology*, *533*(Pt 1), 15–22. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2278598&tool=pmcentrez&rendertype=abstract

Engel, U., & Wolswjk, G. (1996). Oligodendrocyte-Type-2 Astrocyte (0-2A) Progenitor Cells Derived From Adult Rat Spinal Cord : In Vitro Characteristics and Response to PDGF , bFGF and NT-3. *Glia*, *16*(1), 16–26.

Fan, G., Martinowich, K., Chin, M. H., He, F., Fouse, S. D., Hutnick, L., … Sun, Y. E. (2005). DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling. *Development (Cambridge, England)*, *132*(15), 3345–56. doi:10.1242/dev.01912

Fancy, S. P. J., Chan, J. R., Baranzini, S. E., Franklin, R. J. M., & Rowitch, D. H. (2011). Myelin regeneration: a recapitulation of development? *Annual Review of Neuroscience*, *34*, 21–43. doi:10.1146/annurev-neuro-061010-113629

Farrell, B. C., Power, E. M., & McDermott, K. W. (2011). Developmentally regulated expression of Sox9 and microRNAs 124, 128 and 23 in neuroepithelial stem cells in the developing spinal cord. *International Journal of Developmental Neuroscience : The Official Journal of the International Society for Developmental Neuroscience*, *29*(1), 31–6. doi:10.1016/j.ijdevneu.2010.10.001

Fawcett, J. W., & Asher, R. A. (1999). The glial scar and central nervous system repair. *Brain Research Bulletin*, *49*(6), 377–91. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10483914

Field-Fote, E. C., & Tepavac, D. (2002). Improved Intralimb Coordination in People With Incomplete Spinal Cord Injury Following Training With Body Weight Support and Electrical Stimulation. *Physical Therapy*, *82*, 707–715.

Fogarty, M., Richardson, W. D., & Kessaris, N. (2005). A subset of oligodendrocytes generated from radial glia in the dorsal spinal cord. *Development (Cambridge, England)*, *132*(8), 1951–9. doi:10.1242/dev.01777

Fortin, D., Rom, E., Sun, H., Yayon, A., & Bansal, R. (2005). Distinct fibroblast growth factor (FGF)/FGF receptor signaling pairs initiate diverse cellular responses in the oligodendrocyte lineage. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *25*(32), 7470–9. doi:10.1523/JNEUROSCI.2120-05.2005

Frühbeis, C., Fröhlich, D., Kuo, W. P., Amphornrat, J., Thilemann, S., Saab, A. S., … Krämer-Albers, E.-M. (2013). Neurotransmitter-triggered transfer of exosomes mediates oligodendrocyte-neuron communication. *PLoS Biology*, *11*(7), e1001604. doi:10.1371/journal.pbio.1001604

Fu, H., Qi, Y., Tan, M., Cai, J., Takebayashi, H., Nakafuku, M., … Qiu, M. (2002). Dual origin of spinal oligodendrocyte progenitors and evidence for the cooperative role of Olig2 and Nkx2.2 in the control of oligodendrocyte differentiation. *Development (Cambridge, England)*, *129*(3), 681–93. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11830569

Gary, D. S., Malone, M., Capestany, P., Houdayer, T., & McDonald, J. W. (2012). Electrical stimulation promotes the survival of oligodendrocytes in mixed cortical cultures. *Journal of Neuroscience Research*, *90*(1), 72–83. doi:10.1002/jnr.22717

Gorman, P. H., & Mortimer, J. T. (1983). The effect of stimulus parameters on the recruitment characteristics of direct nerve stimulation. *IEEE Transactions on Biomedical Engineering*, *30*(7), 407–414.

Griffin, L., Decker, M. J., Hwang, J. Y., Wang, B., Kitchen, K., Ding, Z., & Ivy, J. L. (2009). *Functional electrical stimulation cycling improves body composition, metabolic and neural factors in persons with spinal cord injury. Journal of electromyography and kinesiology : official journal of the International Society of Electrophysiological Kinesiology* (Vol. 19, pp. 614–622). doi:10.1016/j.jelekin.2008.03.002

Griffiths, I. R., & McCulloch, M. C. (1983). Nerve fibres in spinal cord impact injuries. Part 1. Changes in the myelin sheath during the initial 5 weeks. *Journal of the Neurological Sciences*, *58*(3), 335–49. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6842262

Grill, W. J., & Mortimer, J. T. (1996). The effect of stimulus pulse duration on selectivity of neural stimulation. *IEEE Transactions on Biomedical Engineering*, *43*(2), 161–166.

Grubinska, B., Laszkiewicz, I., Royland, J., Wiggins, R. C., & Konat, G. W. (1994). Differentiation-specific demethylation of myelin associated glycoprotein gene in cultured oligodendrocytes. *Journal of Neuroscience Research*, *39*(3), 233–42. doi:10.1002/jnr.490390302

Hamzaid, N. A., Pithon, K. R., Smith, R. M., & Davis, G. M. (2012). Functional electrical stimulation elliptical stepping versus cycling in spinal cord-injured individuals. *Clinical Biomechanics (Bristol, Avon)*, *27*(7), 731–7. doi:10.1016/j.clinbiomech.2012.03.005

He, X., Yu, Y., Awatramani, R., & Lu, Q. R. (2011). Unwrapping Myelination by MicroRNAs. *The Neuroscientist : A Review Journal Bringing Neurobiology, Neurology and Psychiatry*, *18*(1), 45–55. doi:10.1177/1073858410392382

Hill, R. a, Patel, K. D., Medved, J., Reiss, A. M., & Nishiyama, A. (2013). NG2 cells in white matter but not gray matter proliferate in response to PDGF. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *33*(36), 14558–66. doi:10.1523/JNEUROSCI.2001-12.2013

Hsieh, J., Nakashima, K., Kuwabara, T., Mejia, E., & Gage, F. H. (2004). Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(47), 16659–64. doi:10.1073/pnas.0407643101

Hu, J.-Z., Huang, J.-H., Zeng, L., Wang, G., Cao, M., & Lu, H.-B. (2013). Anti-apoptotic effect of microRNA-21 after contusion spinal cord injury in rats. *Journal of Neurotrauma*, *30*(15), 1349–60. doi:10.1089/neu.2012.2748

Hu, Z., Yu, D., Almeida-Suhett, C., Tu, K., Marini, A. M., Eiden, L., … Li, Z. (2012). Expression of miRNAs and their cooperative regulation of the pathophysiology in traumatic brain injury. *PloS One*, *7*(6), e39357. doi:10.1371/journal.pone.0039357

Hua, Y.-J., Tang, Z.-Y., Tu, K., Zhu, L., Li, Y.-X., Xie, L., & Xiao, H.-S. (2009). Identification and target prediction of miRNAs specifically expressed in rat neural tissue. *BMC Genomics*, *10*, 214. doi:10.1186/1471-2164-10-214

Im, Y. B., Jee, M. K., Choi, J. I., Cho, H. T., Kwon, O. H., & Kang, S. K. (2012). Molecular targeting of NOX4 for neuropathic pain after traumatic injury of the spinal cord. *Cell Death & Disease*, *3*, e426. doi:10.1038/cddis.2012.168

Impey, S., Davare, M., Lesiak, A., Lasiek, A., Fortin, D., Ando, H., … Wayman, G. a. (2010). An activity-induced microRNA controls dendritic spine formation by regulating Rac1-PAK signaling. *Molecular and Cellular Neurosciences*, *43*(1), 146–56. doi:10.1016/j.mcn.2009.10.005

Ioannidou, K., Anderson, K. I., Strachan, D., Edgar, J. M., & Barnett, S. C. (2012). Time-Lapse Imaging of the Dynamics of CNS Glial-Axonal Interactions In Vitro and Ex Vivo. *PloS One*, *7*(1). doi:10.1371/journal.pone.0030775

Ishibashi, T., Dakin, K. a, Stevens, B., Lee, P. R., Kozlov, S. V, Stewart, C. L., & Fields, R. D. (2006). Astrocytes promote myelination in response to electrical impulses. *Neuron*, *49*(6), 823–32. doi:10.1016/j.neuron.2006.02.006

Ishibashi, T., Lee, P. R., Baba, H., & Fields, R. D. (2009). Leukemia inhibitory factor regulates the timing of oligodendrocyte development and myelination in the postnatal optic nerve. *Journal of Neuroscience Research*, *87*(15), 3343–55. doi:10.1002/jnr.22173

Iskandar, B. J., Rizk, E., Meier, B., Hariharan, N., Bottiglieri, T., Finnell, R. H., … Hogan, K. J. (2010). Folate regulation of axonal regeneration in the rodent central nervous system through DNA methylation. *The Journal of Clinical Investigation*, *120*(5), 1603–1616. doi:10.1172/JCI40000.number

Iwamoto, K., Bundo, M., Yamada, K., Takao, H., Iwayama-Shigeno, Y., Yoshikawa, T., & Kato, T. (2005). DNA methylation status of SOX10 correlates with its downregulation and oligodendrocyte dysfunction in schizophrenia. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *25*(22), 5376–81. doi:10.1523/JNEUROSCI.0766-05.2005

Jee, M. K., Jung, J. S., Choi, J. I., Jang, J. A., Kang, K. S., Im, Y. Bin, & Kang, S. K. (2012). MicroRNA 486 is a potentially novel target for the treatment of spinal cord injury. *Brain : A Journal of Neurology*, *135*(Pt 4), 1237–52. doi:10.1093/brain/aws047

Jee, M. K., Jung, J. S., Im, Y. Bin, Jung, S. J., & Kang, S. K. (2012). Silencing of miR20a is crucial for Ngn1-mediated neuroprotection in injured spinal cord. *Human Gene Therapy*, *23*(5), 508–20. doi:10.1089/hum.2011.121

Ji, F., Lv, X., & Jiao, J. (2013). The Role of microRNAs in Neural Stem Cells and Neurogenesis. *Journal of Genetics and Genomics = Yi Chuan Xue Bao*, *40*(2), 61–6. doi:10.1016/j.jgg.2012.12.008

Ji, S., Doucette, J. R., & Nazarali, A. J. (2011). Sirt2 is a novel in vivo downstream target of Nkx 2.2 and enhances oligodendroglial cell differentiation. *Journal of Molecular Cell Biology*, *3*, 351–359.

Johnston, T. E., Modlesky, C. M., Betz, R. R., & Lauer, R. T. (2011). Muscle changes following cycling and/or electrical stimulation in pediatric spinal cord injury. *Archives of Physical Medicine and Rehabilitation*, *92*(12), 1937–43. doi:10.1016/j.apmr.2011.06.031

Juliandi, B., Abematsu, M., & Nakashima, K. (2010). Epigenetic regulation in neural stem cell differentiation. *Development, Growth & Differentiation*, *52*(6), 493–504. doi:10.1111/j.1440-169X.2010.01175.x

Jung, R., Belanger, a, Kanchiku, T., Fairchild, M., & Abbas, J. J. (2009). Neuromuscular stimulation therapy after incomplete spinal cord injury promotes recovery of interlimb coordination during locomotion. *Journal of Neural Engineering*, *6*(5), 055010. doi:10.1088/1741-2560/6/5/055010

Junker, A., Krumbholz, M., Eisele, S., Mohan, H., Augstein, F., Bittner, R., … Meinl, E. (2009). MicroRNA profiling of multiple sclerosis lesions identifies

modulators of the regulatory protein CD47. *Brain : A Journal of Neurology*, *132*(Pt 12), 3342–52. doi:10.1093/brain/awp300

Karimi-Abdolrezaee, S., & Billakanti, R. (2012). Reactive astrogliosis after spinal cord injury-beneficial and detrimental effects. *Molecular Neurobiology*, *46*(2), 251–64. doi:10.1007/s12035-012-8287-4

Krichevsky, A. M., Sonntag, K.-C., Isacson, O., & Kosik, K. S. (2006). Specific microRNAs modulate embryonic stem cell-derived neurogenesis. *Stem Cells (Dayton, Ohio)*, *24*(4), 857–64. doi:10.1634/stemcells.2005-0441

Krishna, V., Konakondla, S., Nicholas, J., Varma, A., Kindy, M., & Wen, X. (2013). Biomaterial-based interventions for neuronal regeneration and functional recovery in rodent model of spinal cord injury: a systematic review. *The Journal of Spinal Cord Medicine*, *36*(3), 174–90. doi:10.1179/2045772313Y.0000000095

Kukley, M., Kiladze, M., Tognatta, R., Hans, M., Swandulla, D., Schramm, J., & Dietrich, D. (2008). Glial cells are born with synapses. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *22*(8), 2957–69. doi:10.1096/fj.07-090985

Kukley, M., Nishiyama, A., & Dietrich, D. (2010). The fate of synaptic input to NG2 glial cells: neurons specifically downregulate transmitter release onto differentiating oligodendroglial cells. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *30*(24), 8320–31. doi:10.1523/JNEUROSCI.0854-10.2010

Lau, P., Verrier, J. D., Nielsen, J. a, Johnson, K. R., Notterpek, L., & Hudson, L. D. (2008). Identification of dynamically regulated microRNA and mRNA networks in developing oligodendrocytes. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *28*(45), 11720–30. doi:10.1523/JNEUROSCI.1932-08.2008

Lee, J.-C., Park, J. H., Yan, B. C., Kim, I. H., Cho, G.-S., Jeoung, D., … Won, M.-H. (2013). Effects of transient cerebral ischemia on the expression of DNA methyltransferase 1 in the gerbil hippocampal CA1 region. *Neurochemical Research*, *38*(1), 74–81. doi:10.1007/s11064-012-0890-2

Lei, P., Li, Y., Chen, X., Yang, S., & Zhang, J. (2009). Microarray based analysis of microRNA expression in rat cerebral cortex after traumatic brain injury. *Brain Research*, *1284*, 191–201. doi:10.1016/j.brainres.2009.05.074

Letzen, B. S., Liu, C., Thakor, N. V, Gearhart, J. D., All, A. H., & Kerr, C. L. (2010). MicroRNA expression profiling of oligodendrocyte differentiation from

human embryonic stem cells. *PloS One*, *5*(5), e10480. doi:10.1371/journal.pone.0010480

Levenson, J. M., Roth, T. L., Lubin, F. D., Miller, C. a, Huang, I.-C., Desai, P., … Sweatt, J. D. (2006). Evidence that DNA (cytosine-5) methyltransferase regulates synaptic plasticity in the hippocampus. *The Journal of Biological Chemistry*, *281*(23), 15763–73. doi:10.1074/jbc.M511767200

Li, J., & Lepski, G. (2013). Cell transplantation for spinal cord injury: a systematic review. *BioMed Research International*, *2013*, 786475. doi:10.1155/2013/786475

Li, J.-S., & Yao, Z.-X. (2012). MicroRNAs: novel regulators of oligodendrocyte differentiation and potential therapeutic targets in demyelination-related diseases. *Molecular Neurobiology*, *45*(1), 200–12. doi:10.1007/s12035-011-8231-z

Li, Q., Brus-Ramer, M., Martin, J. H., & McDonald, J. W. (2010). Electrical stimulation of the medullary pyramid promotes proliferation and differentiation of oligodendrocyte progenitor cells in the corticospinal tract of the adult rat. *Neuroscience Letters*, *479*(2), 128–33. doi:10.1016/j.neulet.2010.05.043

Lin, S., Huang, Y., Zhang, L., Heng, M. Y., Ptácek, L. J., & Fu, Y.-H. (2013). MicroRNA-23a promotes myelination in the central nervous system. *PNAS*, *110*(43), 17468–17473. doi:10.1073/pnas.1317182110/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1317182110

Lin, S.-T., & Fu, Y.-H. (2009). miR-23 regulation of lamin B1 is crucial for oligodendrocyte development and myelination. *Disease Models & Mechanisms*, *2*(3-4), 178–88. doi:10.1242/dmm.001065

Liu, B., Chen, X., Wang, Z. Q., & Tong, W. M. (2014). DNA damage and oxidative injury are associated with hypomyelination in the corpus callosum of newborn Nbn(CNS-del) mice. *Journal of Neuroscience Research*, *92*(2), 254–66. doi:10.1002/jnr.23313

Liu, G., Detloff, M. R., Miller, K. N., Santi, L., & Houlé, J. D. (2012). Exercise modulates microRNAs that affect the PTEN/mTOR pathway in rats after spinal cord injury. *Experimental Neurology*, *233*(1), 447–56. doi:10.1016/j.expneurol.2011.11.018

Liu, G., Keeler, B. E., Zhukareva, V., & Houlé, J. D. (2010). Cycling exercise affects the expression of apoptosis-associated microRNAs after spinal cord injury in rats. *Experimental Neurology*, *226*(1), 200–6. doi:10.1016/j.expneurol.2010.08.032

Liu, H., Hu, Q., D'ercole, a J., & Ye, P. (2009). Histone deacetylase 11 regulates oligodendrocyte-specific gene expression and cell development in OL-1 oligodendroglia cells. *Glia*, *57*(1), 1–12. doi:10.1002/glia.20729

Liu, H., Wu, H., Wang, Y., Wang, Y., Wu, X., Ju, S., & Wang, X. (2012). Inhibition of class II histone deacetylase blocks proliferation and promotes neuronal differentiation of the embryonic rat neural progenitor cells. *Acta Neurobiologiae Experimentalis*, *72*(4), 365–76. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23377267

Liu, N.-K., Wang, X.-F., Lu, Q.-B., & Xu, X.-M. (2009). Altered MicroRNA Expression Following Traumatic Spinal Cord Injury. *Experimental Neurology*, *219*(2), 424–429. doi:10.1016/j.expneurol.2009.06.015.Altered

Liu, N.-K., & Xu, X.-M. (2011). MicroRNA in central nervous system trauma and degenerative disorders. *Physiological Genomics*, *43*(10), 571–80. doi:10.1152/physiolgenomics.00168.2010

Liu, X. S., Chopp, M., Kassis, H., Jia, L. F., Hozeska-Solgot, a, Zhang, R. L., … Zhang, Z. G. (2012). Valproic acid increases white matter repair and neurogenesis after stroke. *Neuroscience*, *220*, 313–21. doi:10.1016/j.neuroscience.2012.06.012

Liu, X. S., Chopp, M., Wang, X. L., Zhang, L., Hozeska-Solgot, A., Tang, T., … Zhang, Z. G. (2013). MicroRNA-17-92 cluster mediates the proliferation and survival of neural progenitor cells after stroke. *The Journal of Biological Chemistry*, *288*(18), 12478–88. doi:10.1074/jbc.M112.449025

Liu, X. S., Chopp, M., Zhang, R. L., Tao, T., Wang, X. L., Kassis, H., … Zhang, Z. G. (2011). MicroRNA profiling in subventricular zone after stroke: MiR-124a regulates proliferation of neural progenitor cells through Notch signaling pathway. *PloS One*, *6*(8), e23461. doi:10.1371/journal.pone.0023461

Liu, X. Z., Xu, X. M., Hu, R., Du, C., Zhang, S. X., McDonald, J. W., … Choi, D. W. (1997). Neuronal and glial apoptosis after traumatic spinal cord injury. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *17*(14), 5395–406. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9204923

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.)*, *25*(4), 402–8. doi:10.1006/meth.2001.1262

Lu, W.-H., Wang, C.-Y., Chen, P.-S., Wang, J.-W., Chuang, D.-M., Yang, C.-S., & Tzeng, S.-F. (2013). Valproic acid attenuates microgliosis in injured spinal

cord and purinergic P2X4 receptor expression in activated microglia. *Journal of Neuroscience Research*, *91*(5), 694–705. doi:10.1002/jnr.23200

Lundberg, J., Karimi, M., von Gertten, C., Holmin, S., Ekström, T. J., & Sandberg-Nordqvist, A.-C. (2009). Traumatic brain injury induces relocalization of DNA-methyltransferase 1. *Neuroscience Letters*, *457*(1), 8–11. doi:10.1016/j.neulet.2009.03.105

Luo, S., Zhang, X., Yu, M., Yan, H., Liu, H., Wilson, J. X., & Huang, G. (2013). Folic acid acts through DNA methyltransferases to induce the differentiation of neural stem cells into neurons. *Cell Biochemistry and Biophysics*, *66*(3), 559–66. doi:10.1007/s12013-012-9503-6

Lv, L., Han, X., Sun, Y., Wang, X., & Dong, Q. (2012). Valproic acid improves locomotion in vivo after SCI and axonal growth of neurons in vitro. *Experimental Neurology*, *233*(2), 783–90. doi:10.1016/j.expneurol.2011.11.042

Lv, L., Sun, Y., Han, X., Xu, C., Tang, Y.-P., & Dong, Q. (2011). Valproic acid improves outcome after rodent spinal cord injury: potential roles of histone deacetylase inhibition. *Brain Research*, *1396*, 60–8. doi:10.1016/j.brainres.2011.03.040

Lyssiotis, C. a, Walker, J., Wu, C., Kondo, T., Schultz, P. G., & Wu, X. (2007). Inhibition of histone deacetylase activity induces developmental plasticity in oligodendrocyte precursor cells. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(38), 14982–7. doi:10.1073/pnas.0707044104

MacDonald, J. L., & Roskams, a. J. (2009). Epigenetic regulation of nervous system development by DNA methylation and histone deacetylation. *Progress in Neurobiology*, *88*(3), 170–183. doi:10.1016/j.pneurobio.2009.04.002

Madathil, S. K., Nelson, P. T., Saatman, K. E., & Wilfred, B. R. (2011). MicroRNAs in CNS injury: potential roles and therapeutic implications. *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology*, *33*(1), 21–6. doi:10.1002/bies.201000069

Magri, L., Swiss, V. a., Jablonska, B., Lei, L., Pedre, X., Walsh, M., … Casaccia, P. (2014). E2F1 Coregulates Cell Cycle Genes and Chromatin Components during the Transition of Oligodendrocyte Progenitors from Proliferation to Differentiation. *Journal of Neuroscience*, *34*(4), 1481–1493. doi:10.1523/JNEUROSCI.2840-13.2014

Makeyev, E. V, Zhang, J., Carrasco, M. a, & Maniatis, T. (2007). The microRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. *Molecular Cell*, *27*(3), 435–48. doi:10.1016/j.molcel.2007.07.015

Malone, M., Gary, D., Yang, I. H., Miglioretti, A., Houdayer, T., Thakor, N., & McDonald, J. (2013). Neuronal activity promotes myelination via a cAMP pathway. *Glia*, 1–12. doi:10.1002/glia.22476

Martin, L. J., & Wong, M. (2013). Aberrant regulation of DNA methylation in amyotrophic lateral sclerosis: a new target of disease mechanisms. *Neurotherapeutics : The Journal of the American Society for Experimental NeuroTherapeutics*, *10*(4), 722–33. doi:10.1007/s13311-013-0205-6

McDonald, J. W., Althomsons, S. P., Hyrc, K. L., Choi, D. W., & Goldberg, M. P. (1998). Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity. *Nature Medicine*, *4*(3), 291–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9500601

McDonald, J. W., Shapiro, S. M., Silverstein, F. S., & Johnston, M. V. (1998). Role of glutamate receptor-mediated excitotoxicity in bilirubin-induced brain injury in the Gunn rat model. *Experimental Neurology*, *150*(1), 21–9. doi:10.1006/exnr.1997.6762

McKinnon, R. D., Waldron, S., & Kiel, M. E. (2005). PDGF alpha-receptor signal strength controls an RTK rheostat that integrates phosphoinositol 3'-kinase and phospholipase Cgamma pathways during oligodendrocyte maturation. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *25*(14), 3499–508. doi:10.1523/JNEUROSCI.5049-04.2005

Mekhail, M., Almazan, G., & Tabrizian, M. (2012). Oligodendrocyte-protection and remyelination post-spinal cord injuries: a review. *Progress in Neurobiology*, *96*(3), 322–39. doi:10.1016/j.pneurobio.2012.01.008

Mitew, S., Hay, C. M., Peckham, H., Xiao, J., Koenning, M., & Emery, B. (2013). Mechanisms regulating the development of oligodendrocytes and central nervous system myelin. *Neuroscience*. doi:10.1016/j.neuroscience.2013.11.029

Morrison, K. E., Rodgers, a B., Morgan, C. P., & Bale, T. L. (2013). Epigenetic mechanisms in pubertal brain maturation. *Neuroscience*. doi:10.1016/j.neuroscience.2013.11.014

Nakanishi, K., Nakasa, T., Tanaka, N., Ishikawa, M., Yamada, K., Yamasaki, K., … Ochi, M. (2010). Responses of microRNAs 124a and 223 following spinal cord injury in mice. *Spinal Cord*, *48*(3), 192–6. doi:10.1038/sc.2009.89

Niu, H., Wang, K., Zhang, A., Yang, S., Song, Z., Wang, W., … Wang, Y. (2012). miR-92a is a critical regulator of the apoptosis pathway in glioblastoma with inverse expression of BCL2L11. *Oncology Reports*, *28*(5), 1771–7. doi:10.3892/or.2012.1970

Norenberg, M. D., Smith, J., & Marcillo, A. (2004). The pathology of human spinal cord injury: defining the problems. *Journal of Neurotrauma*, *21*(4), 429–40. doi:10.1089/089771504323004575

Nudelman, A. S., DiRocco, D. P., Lambert, T. J., Garelick, M. G., Le, J., Nathanson, N. M., & Storm, D. R. (2010). Neuronal activity rapidly induces transcription of the CREB-regulated microRNA-132, in vivo. *Hippocampus*, *20*(4), 492–8. doi:10.1002/hipo.20646

Nwaobi, S. E., Lin, E., Peramsetty, S. R., & Olsen, M. L. (2014). DNA methylation functions as a critical regulator of Kir4.1 expression during CNS development. *Glia*, *62*(3), 411–27. doi:10.1002/glia.22613

Pazhoohan, S., Satarian, L., Asghari, A.-A., Salimi, M., Kiani, S., Mani, A.-R., & Javan, M. (2014). Valproic Acid attenuates disease symptoms and increases endogenous myelin repair by recruiting neural stem cells and oligodendrocyte progenitors in experimental autoimmune encephalomyelitis. *Neuro-Degenerative Diseases*, *13*(1), 45–52. doi:10.1159/000352021

Penas, C., Verdú, E., Asensio-Pinilla, E., Guzmán-Lenis, M. S., Herrando-Grabulosa, M., Navarro, X., & Casas, C. (2011). Valproate reduces CHOP levels and preserves oligodendrocytes and axons after spinal cord injury. *Neuroscience*, *178*, 33–44. doi:10.1016/j.neuroscience.2011.01.012

Pfeiffer, S. E., Warrington, a E., & Bansal, R. (1993). The oligodendrocyte and its many cellular processes. *Trends in Cell Biology*, *3*(6), 191–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14731493

Profyris, C., Cheema, S. S., Zang, D., Azari, M. F., Boyle, K., & Petratos, S. (2004). Degenerative and regenerative mechanisms governing spinal cord injury. *Neurobiology of Disease*, *15*(3), 415–36. doi:10.1016/j.nbd.2003.11.015

Pusic, A. D., & Kraig, R. P. (2014). Youth and environmental enrichment generate serum exosomes containing miR-219 that promote CNS myelination. *Glia*, *62*(2), 284–99. doi:10.1002/glia.22606

Rabchevsky, A. G., Sullivan, P. G., & Scheff, S. W. (2007). Temporal-Spatial Dynamics in Oligodendrocyte and Glial Progenitor Cell Numbers Throughout Ventrolateral White Matter Following Contusion Spinal Cord Injury. *Glia*, *55*, 831–843. doi:10.1002/glia

Redell, J. B., Moore, A. N., Ward, N. H., Hergenroeder, G. W., & Dash, P. K. (2010). Human traumatic brain injury alters plasma microRNA levels. *Journal of Neurotrauma*, *27*(12), 2147–56. doi:10.1089/neu.2010.1481

Rivera, F. J., Steffenhagen, C., Kremer, D., Kandasamy, M., Sandner, B., Couillard-Despres, S., … Aigner, L. (2010). Deciphering the oligodendrogenic program of neural progenitors: cell intrinsic and extrinsic regulators. *Stem Cells and Development*, *19*(5), 595–606. doi:10.1089/scd.2009.0293

Rivero-Melián, C. (1996). Organization of hindlimb nerve projections to the rat spinal cord: a choleragenoid horseradish peroxidase study. *The Journal of Comparative Neurology*, *364*(4), 651–63. doi:10.1002/(SICI)1096-9861(19960122)364:4<651::AID-CNE4>3.0.CO;2-2

Ryan, K. M., Donovan, S. M. O., & McLoughlin, D. M. (2013). Electroconvulsive stimulation alters levels of BDNF-associated microRNAs. *Neuroscience Letters*, 1–5. doi:10.1016/j.neulet.2013.05.035

Ryge, J., Winther, O., Wienecke, J., Sandelin, A., Westerdahl, A.-C., Hultborn, H., & Kiehn, O. (2010). Transcriptional regulation of gene expression clusters in motor neurons following spinal cord injury. *BMC Genomics*, *11*, 365. doi:10.1186/1471-2164-11-365

Sadowsky, C. L., Hammond, E. R., Strohl, A. B., Commean, P. K., Eby, S. a, Damiano, D. L., … McDonald Iii, J. W. (2013). Lower extremity functional electrical stimulation cycling promotes physical and functional recovery in chronic spinal cord injury. *The Journal of Spinal Cord Medicine*, *0*(0), 1–9. doi:10.1179/2045772313Y.0000000101

Sharifi, M., Salehi, R., Gheisari, Y., & Kazemi, M. (2013). Inhibition of MicroRNA miR-92a Inhibits Cell Proliferation in Human Acute Promyelocytic Leukemia. *Turkish Journal of Haematology : Official Journal of Turkish Society of Haematology*, *30*(2), 157–62. doi:10.4274/Tjh.2012.0171

Sharifi, M., Salehi, R., Gheisari, Y., & Kazemi, M. (2014). Inhibition of microRNA miR-92a induces apoptosis and inhibits cell proliferation in human acute promyelocytic leukemia through modulation of p63 expression. *Molecular Biology Reports*. doi:10.1007/s11033-014-3134-5

Shein, N. a, & Shohami, E. (2011). Histone deacetylase inhibitors as therapeutic agents for acute central nervous system injuries. *Molecular Medicine (Cambridge, Mass.)*, *17*(5-6), 448–56. doi:10.2119/molmed.2011.00038

Shen, S., & Casaccia-Bonnefil, P. (2008). Post-translational modifications of nucleosomal histones in oligodendrocyte lineage cells in development and

disease. *Journal of Molecular Neuroscience : MN*, *35*(1), 13–22. doi:10.1007/s12031-007-9014-x

Shen, S., Li, J., & Casaccia-Bonnefil, P. (2005). Histone modifications affect timing of oligodendrocyte progenitor differentiation in the developing rat brain. *The Journal of Cell Biology*, *169*(4), 577–89. doi:10.1083/jcb.200412101

Shen, S., Liu, A., Li, J., Wolubah, C., & Casaccia-Bonnefil, P. (2008). Epigenetic memory loss in aging oligodendrocytes in the corpus callosum. *Neurobiology of Aging*, *29*(3), 452–63. doi:10.1016/j.neurobiolaging.2006.10.026

Shen, S., Sandoval, J., Swiss, V. a, Li, J., Dupree, J., Franklin, R. J. M., & Casaccia-Bonnefil, P. (2008). Age-dependent epigenetic control of differentiation inhibitors is critical for remyelination efficiency. *Nature Neuroscience*, *11*(9), 1024–34. doi:10.1038/nn.2172

Shin, D., Shin, J.-Y., McManus, M. T., Ptácek, L. J., & Fu, Y.-H. (2009). Dicer ablation in oligodendrocytes provokes neuronal impairment in mice. *Annals of Neurology*, *66*(6), 843–57. doi:10.1002/ana.21927

Sim, F. J., Zhao, C., Penderis, J., & Franklin, R. J. M. (2002). The Age-Related Decrease in CNS Remyelination Efficiency Is Attributable to an Impairment of Both Oligodendrocyte Progenitor Recruitment and Differentiation. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *22*(7), 2451–2459.

Simmons, R. K., Stringfellow, S. a, Glover, M. E., Wagle, A. a, & Clinton, S. M. (2013). DNA methylation markers in the postnatal developing rat brain. *Brain Research*, *1533*, 26–36. doi:10.1016/j.brainres.2013.08.005

Simon, C., Götz, M., & Dimou, L. (2011). Progenitors in the adult cerebral cortex: cell cycle properties and regulation by physiological stimuli and injury. *Glia*, *59*(6), 869–81. doi:10.1002/glia.21156

Simpson, P. B., & Armstrong, R. C. (1999). Intracellular signals and cytoskeletal elements involved in oligodendrocyte progenitor migration. *Glia*, *26*(1), 22–35. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10088669

Stevens, B., Porta, S., Haak, L. L., Gallo, V., & Fields, R. D. (2002). Adenosine: a neuron-glial transmitter promoting myelination in the CNS in response to action potentials. *Neuron*, *36*(5), 855–68. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1201407&tool=pmcentrez&rendertype=abstract

Stevens, B., Tanner, S., & Fields, R. D. (1998). Control of myelination by specific patterns of neural impulses. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *18*(22), 9303–11. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9801369

Strickland, E. R., Hook, M. a, Balaraman, S., Huie, J. R., Grau, J. W., & Miranda, R. C. (2011). MicroRNA dysregulation following spinal cord contusion: implications for neural plasticity and repair. *Neuroscience*, *186*, 146–60. doi:10.1016/j.neuroscience.2011.03.063

Swett, J. E., & Woolf, C. J. (1985). The somatotopic organization of primary afferent terminals in the superficial laminae of the dorsal horn of the rat spinal cord. *The Journal of Comparative Neurology*, *231*(1), 66–77. doi:10.1002/cne.902310106

Sylvestre, Y., De Guire, V., Querido, E., Mukhopadhyay, U. K., Bourdeau, V., Major, F., … Chartrand, P. (2007). An E2F/miR-20a autoregulatory feedback loop. *The Journal of Biological Chemistry*, *282*(4), 2135–43. doi:10.1074/jbc.M608939200

Tian, L., Fang, Y., Xue, J., & Chen, J. (2013). Four microRNAs promote prostate cell proliferation with regulation of PTEN and its downstream signals in vitro. *PloS One*, *8*(9), e75885. doi:10.1371/journal.pone.0075885

Totoiu, M. O., & Keirstead, H. S. (2005). Spinal cord injury is accompanied by chronic progressive demyelination. *The Journal of Comparative Neurology*, *486*(4), 373–83. doi:10.1002/cne.20517

Trapp, B. D., Nishiyama, a, Cheng, D., & Macklin, W. (1997). Differentiation and death of premyelinating oligodendrocytes in developing rodent brain. *The Journal of Cell Biology*, *137*(2), 459–68. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2139778&tool=pmcentrez&rendertype=abstract

Tripathi, R. B., Clarke, L. E., Burzomato, V., Kessaris, N., Anderson, P. N., Attwell, D., & Richardson, W. D. (2011). Dorsally and ventrally derived oligodendrocytes have similar electrical properties but myelinate preferred tracts. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *31*(18), 6809–19. doi:10.1523/JNEUROSCI.6474-10.2011

Udina, E., Furey, M., Busch, S., Silver, J., Gordon, T., & Fouad, K. (2008). Electrical stimulation of intact peripheral sensory axons in rats promotes outgrowth of their central projections. *Experimental Neurology*, *210*(1), 238–47. doi:10.1016/j.expneurol.2007.11.007

Visvanathan, J., Lee, S., Lee, B., Lee, J. W., & Lee, S.-K. (2007). The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development. *Genes & Development*, *21*(7), 744–9. doi:10.1101/gad.1519107

Wake, H., Lee, P. R., & Fields, R. D. (2011). Control of local protein synthesis and initial events in myelination by action potentials. *Science (New York, N.Y.)*, *333*(6049), 1647–51. doi:10.1126/science.1206998

Wan, L., Xia, R., & Ding, W. (2010). Short-term low-frequency electrical stimulation enhanced remyelination of injured peripheral nerves by inducing the promyelination effect of brain-derived neurotrophic factor on Schwann cell polarization. *Journal of Neuroscience Research*, *88*(12), 2578–87. doi:10.1002/jnr.22426

Wang, E., & Cambi, F. (2012). MicroRNA expression in mouse oligodendrocytes and regulation of proteolipid protein gene expression. *Journal of Neuroscience Research*, *90*(9), 1701–12. doi:10.1002/jnr.23055

Wanner, I. B., Anderson, M. a, Song, B., Levine, J., Fernandez, A., Gray-Thompson, Z., … Sofroniew, M. V. (2013). Glial scar borders are formed by newly proliferated, elongated astrocytes that interact to corral inflammatory and fibrotic cells via STAT3-dependent mechanisms after spinal cord injury. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *33*(31), 12870–86. doi:10.1523/JNEUROSCI.2121-13.2013

Watkins, T., Emery, B., Mulinyawe, S., & Barres, B. (2008). Distinct stages of myelination regulated by gamma-secretase and astrocytes in rapidly myelinating CNS coculture system. *Neuron*, *60*(4), 555–569. doi:10.1016/j.neuron.2008.09.011.Distinct

Wibrand, K., Panja, D., Tiron, A., Ofte, M. L., Skaftnesmo, K.-O., Lee, C. S., … Bramham, C. R. (2010). Differential regulation of mature and precursor microRNA expression by NMDA and metabotropic glutamate receptor activation during LTP in the adult dentate gyrus in vivo. *The European Journal of Neuroscience*, *31*(4), 636–45. doi:10.1111/j.1460-9568.2010.07112.x

Willemen, H. L. D. M., Huo, X.-J., Mao-Ying, Q.-L., Zijlstra, J., Heijnen, C. J., & Kavelaars, A. (2012). MicroRNA-124 as a novel treatment for persistent hyperalgesia. *Journal of Neuroinflammation*, *9*, 143. doi:10.1186/1742-2094-9-143

Wilson, J. R., Forgione, N., & Fehlings, M. G. (2013). Emerging therapies for acute traumatic spinal cord injury. *CMAJ : Canadian Medical Association*

Journal = Journal de l'Association Medicale Canadienne, *185*(6), 485–92. doi:10.1503/cmaj.121206

Wolswjk, G., & Noble, M. (1992). Cooperation Between PDGF and FGF Converts Slowly Dividing O-2A adutt Progenitor Cells to Rapidly Dividing Cells with Characteristics of O _ 2A " nat Progenitor Cells. *The Journal of Cell Biology*, *118*(4), 889–900.

Wood, T. L., Bercury, K. K., Cifelli, S. E., Mursch, L. E., Min, J., Dai, J., & Macklin, W. B. (2013). mTOR: a link from the extracellular milieu to transcriptional regulation of oligodendrocyte development. *ASN Neuro*, *5*(1), e00108. doi:10.1042/AN20120092

Wu, M., Hernandez, M., Shen, S., Sabo, J. K., Kelkar, D., Wang, J., … Casaccia, P. (2012). Differential modulation of the oligodendrocyte transcriptome by sonic hedgehog and bone morphogenetic protein 4 via opposing effects on histone acetylation. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *32*(19), 6651–64. doi:10.1523/JNEUROSCI.4876-11.2012

Wu, Q., Yang, Z., Wang, F., Hu, S., Yang, L., Shi, Y., & Fan, D. (2013). MiR-19b/20a/92a regulates the self-renewal and proliferation of gastric cancer stem cells. *Journal of Cell Science*, *126*(Pt 18), 4220–9. doi:10.1242/jcs.127944

Wu, Z., Huang, K., Yu, J., Le, T., Namihira, M., Liu, Y., … Fan, G. (2012). Dnmt3a regulates both proliferation and differentiation of mouse neural stem cells. *Journal of Neuroscience Research*, *90*(10), 1883–91. doi:10.1002/jnr.23077

Xu, W., Wang, X., Li, P., Qin, K., & Jiang, X. (2012). miR-124 regulates neural stem cells in the treatment of spinal cord injury. *Neuroscience Letters*, *529*(1), 12–7. doi:10.1016/j.neulet.2012.09.025

Ye, F., Chen, Y., Hoang, T., Montgomery, R. L., Zhao, X., Bu, H., … Lu, Q. R. (2009). HDAC1 and HDAC2 regulate oligodendrocyte differentiation by disrupting the beta-catenin-TCF interaction. *Nature Neuroscience*, *12*(7), 829–38. doi:10.1038/nn.2333

Yoo, A. S., Staahl, B. T., Chen, L., & Crabtree, G. R. (2009). MicroRNA-mediated switching of chromatin-remodelling complexes in neural development. *Nature*, *460*(7255), 642–6. doi:10.1038/nature08139

Young, K. M., Psachoulia, K., Tripathi, R. B., Dunn, S.-J., Cossell, L., Attwell, D., … Richardson, W. D. (2013). Oligodendrocyte dynamics in the healthy adult

CNS: evidence for myelin remodeling. *Neuron*, *77*(5), 873–85. doi:10.1016/j.neuron.2013.01.006

Yuan, Y.-M., & He, C. (2013). The glial scar in spinal cord injury and repair. *Neuroscience Bulletin*, *29*(4), 421–35. doi:10.1007/s12264-013-1358-3

Yunta, M., Nieto-Díaz, M., Esteban, F. J., Caballero-López, M., Navarro-Ruíz, R., Reigada, D., … Maza, R. M. (2012). MicroRNA dysregulation in the spinal cord following traumatic injury. *PloS One*, *7*(4), e34534. doi:10.1371/journal.pone.0034534

Zhang, B., West, E. J., Van, K. C., Gurkoff, G. G., Zhou, J., Zhang, X.-M., … Lyeth, B. G. (2008). HDAC inhibitor increases histone H3 acetylation and reduces microglia inflammatory response following traumatic brain injury in rats. *Brain Research*, *1226*, 181–91. doi:10.1016/j.brainres.2008.05.085

Zhang, Z.-Y., Zhang, Z., Fauser, U., & Schluesener, H. J. (2007). Global hypomethylation defines a sub-population of reactive microglia/macrophages in experimental traumatic brain injury. *Neuroscience Letters*, *429*(1), 1–6. doi:10.1016/j.neulet.2007.09.061

Zhao, X., He, X., Han, X., Yu, Y., Ye, F., Chen, Y., … Lu, Q. R. (2010). MicroRNA-mediated control of oligodendrocyte differentiation. *Neuron*, *65*(5), 612–26. doi:10.1016/j.neuron.2010.02.018

Ziu, M., Fletcher, L., Savage, J. G., Jimenez, D. F., Digicaylioglu, M., & Bartanusz, V. (2013). Spatial and temporal expression levels of specific microRNAs in a spinal cord injury mouse model and their relationship to the duration of compression. *The Spine Journal : Official Journal of the North American Spine Society*, *1*, 1–8. doi:10.1016/j.spinee.2013.08.015

Zuchero, J. B., & Barres, B. a. (2013). Intrinsic and extrinsic control of oligodendrocyte development. *Current Opinion in Neurobiology*, *23*(6), 914–20. doi:10.1016/j.conb.2013.06.005

Curriculum Vitae

Amber T. Ballard **March 26, 2014**

Education

- **Ph.D. in Biomedical Engineering (2014).** Johns Hopkins University School of Medicine (Baltimore, MD). Advisors: John McDonald, M.D., Ph.D. and Andres Hurtado, M.D. Dissertation Title: The Effect of Functional Electrical Stimulation on Oligodendrocyte Biology after Spinal Cord Injury
- **B.S. in Chemical Engineering (2005).** University of Maryland, Baltimore County (Baltimore, MD).

Professional Experience

- **Ph.D. candidate** (**2007 – present).** International Center for Spinal Cord Injury. Hugo W. Moser Research Institute at Kennedy Krieger (Baltimore, MD). Advisors: John McDonald, M.D., Ph.D. and Andres Hurtado, M.D.
- **Undergraduate student researcher (2004 – 2005).** Department of Biomedical Engineering, Johns Hopkins University (Baltimore, MD). Advisor: Reza Shadmehr, Ph.D.
- **Undergraduate student researcher (2004).** Department of Biomedical Engineering, Case Western Reserve University (Cleveland, OH). Advisor: Dustin Tyler, Ph.D.
- **Undergraduate student researcher (2003).** Department of Bioengineering, University of California, Berkeley (Berkeley, CA). Advisor: Lisa Pruitt, Ph.D.
- **Undergraduate student researcher (2002).** Department of Pharmacology of Experimental Therapeutics, University of Maryland (Baltimore, MD). Advisor: Mohyee Eldefrawi, Ph.D.

Honors and Awards

- **2004 – 2005.** MARC Undergraduate Student Training in Academic Research (U*STAR) Scholarship. University of Maryland, Baltimore County (Baltimore, MD).

- **2000 – 2003.** Meyerhoff Scholarship. University of Maryland, Baltimore County (Baltimore, MD).

Presentations

- **Amber Ballard**, Andres Hurtado, Visar Belegu, John McDonald. (2013) *microRNA expression in central nervous system development and injury*. Oral presentation at the International Center for Spinal Cord Injury, Kennedy Krieger Institute (Baltimore, MD).
- **Amber Ballard**, Andres Hurtado, John McDonald. (2013) *Functional electrical stimulation after chronic spinal cord injury*. Oral presentation at the International Center for Spinal Cord Injury, Kennedy Krieger Institute (Baltimore, MD).
- Andres Hurtado, **Amber T. (Ballard) Cross**, Devin S. Gary, Misti Malone, Visar Belegu, Dane F. Wendell, Yun Qu, Thierry Houdayer, John W. McDonald. (2011) *Effect of functional electrical stimulation on myelination after complete rat spinal cord transection*. Poster presented at the Society for Neuroscience conference (Washington, DC).
- **Amber (Ballard) Cross**, Andres Hurtado, John McDonald. (2011) *Functional electrical stimulation after spinal cord injury in adult rodents*. Oral presentation at the Department of Biomedical Engineering, Johns Hopkins University (Baltimore, MD).
- Andres Hurtado, **Amber T. (Ballard) Cross**, Dane F. Wendell, Yun Qu, Thierry Houdayer, John W. McDonald. (2010) *Effect of functional electrical stimulation frequency on cell birth in a complete transection rat model of spinal cord injury*. Poster presented at the Society for Neuroscience conference (San Diego, CA).
- **Amber Ballard** and Dustin Tyler. (2004) *Topographic Anatomy of the Nerves of the Larynx and Tongue.* Poster presented at the Annual Biomedical Research Conference for Minority Students (ABRCMS; Dallas, TX).
- **Amber Ballard** and Dustin Tyler. (2004) *Topographic Anatomy of the Nerves of the Larynx and Tongue.* Poster presented at the SPUR Annual Poster Presentation. Case Western Reserve University (Cleveland, OH).
- **Amber Ballard**, Ayyana Chakravartula, Lisa Pruitt. (2003) *Near-surface viscoelastic properties of ultra-high molecular weight polyethylene.* Oral presentation at the Annual Guidant/SUPERB Research Symposium. University of California, Berkeley (Berkeley, CA).
- **Amber Ballard** and Mohyee Eldefrawi. (2002) *Ceftiofur fiber optic biosensor.* Poster presented at the University of Maryland Medical Center Annual Student Research Forum (Baltimore, MD).

Leadership Experience

- **2007 – present.** Training students and research assistants in the International Center for Spinal Cord Injury, Hugo W. Moser Research Institute at Kennedy Krieger (Baltimore, MD).
- **2011 – 2012.** Founder and Chair of the Academic Academy, Incentive Mentoring Program (Baltimore, MD).
- **2007 – 2011.** Director of the Academic Affairs Committee, Incentive Mentoring Program (Baltimore, MD).
- **2009.** Teaching assistant, Molecules and Cells, Department of Biomedical Engineering, Johns Hopkins University (Baltimore, MD).
- **2008.** Teaching assistant, Statistical Mechanics and Thermodynamics, Department of Biomedical Engineering, Johns Hopkins University (Baltimore, MD).
- **2007 – 2008.** Head of household, Incentive Mentoring Program (Baltimore, MD).
- **2006 – 2007.** Mentor, Incentive Mentoring Program (Baltimore, MD).